# Unravelling coexistence of cryptic *Litoditis marina* species

ISBN: 9789082561104

Ghent University

Faculty of Sciences

Biology Department

Marine Biology Research Group

Campus De Sterre – S8

Krijgslaan 281

9000 Gent

Belgium

Printed by:

Reproduct NV

Voskenslaan 205

9000 Gent

Lay-out: Nele De Meester, pictures adapted from Annelien Rigaux, Ruth Gingold, Sofie Derycke and Worms (Team17 Digital Ltd)


Academic year 2015 – 2016

Defended in public on June 30th, 2016

Co-authored one or more chapters:

Holly Bik, Dries Bonte, Simon Creer, Sofie Derycke, Giovanni Dos Santos, Ruth Gingold, Tom Moens, Annelien Rigaux, Kelley Thomas and Yirina Valdes

For the citation of published work reprinted in this thesis, please refer to the original publication, as mentioned in the beginning of each chapter.

To refer to this thesis, please cite as:

De Meester N. (2016) Coexistence of cryptic *Litoditis marina* species. Ghent University, 334 pp.

Nele De Meester

# Unravelling coexistence of cryptic *Litoditis marina* species

# Ontwarren van de co-existentie van cryptische *Litoditis marina* soorten

PROMOTOR: Prof. Dr. Tom Moens

COPROMOTOR: Prof. Dr. Sofie Derycke

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor in Science (Biology)

Academic year: 2015 - 2016

*As we know, there are known knowns.*

*There are things we know we know.*

*We also know there are known unknowns.*

*That is to say, we know there are some things we do not know.*

*But there are also unknown unknowns.*

*The ones we don’t know we don't know.*

*(Donald Rumsfeld)*

**MEMBERS OF THE EXAMINATION COMMITTEE:**

Prof. Dr. Tom Moens (Ghent University): promotor

Prof. Dr. Sofie Derycke (Royal Belgian Institute of Natural Sciences): co-promotor

Prof. Dr. Koen Sabbe (Ghent University): chair

Prof Dr. Dries Bonte (Ghent University): secretary

Prof. Dr. Gustavo Fonseca (Federal University of São Paulo)

Prof. Dr. Walter Sudhaus (Free University Berlin)

Prof. Dr. Jan Vanaverbeke (Royal Belgian Institute of Natural Sciences)

Prof. Dr. Ann Vanreusel (Ghent University)

Prof. Dr. Magda Vincx (Ghent University)

**Defended in public on June 30th, 2016**

# Table of contents

# DANKWOORD

"Moeke, **later word ik ecoloog**!", deze woorden sprak ik als 9-jarig Neleke uit. Mijn moeder keek even vreemd op, zag dat ik in mijn honderdste boek van de natuur aan het lezen was en antwoordde dan maar "Dat moet je zeker doen!". En kijk: 20 jaar later kan ik met heel veel trots zeggen dat ik me echt een ecoloog voel, na het indienen van dit boekje!

Al is het zeker niet altijd zo evident geweest. Natuurlijk wou ik graag iets met biologie doen, maar gewoon uit interesse. In werken? Nee hoor! Ik ging na de biologie wel nog iets anders gaan studeren, iets waar ik echt wou in gaan werken. Iets met kinderen of jongeren, iets waar je creatief in kon zijn, iets waar ik me echt nuttig voelde! Het CLB en mijn medeleerlingen in het zesde middelbaar bekrachtigden dit: "Nele, jij moet iets sociaal gaan doen! Pedagogie, onderwijs, keuze genoeg!". Maar de roep van de wetenschap was te luid en alles viel op zijn plaats in 2009 als ik deze job als assistent kon beginnen.

Want ja, nu kon ik én een wetenschapper worden én lesgeven. Mijn twee passies mooi door elkaar geweven. Een betere job kon ik me niet inbeelden. En ook al keken mijn medestudenten vreemd als ik zei dat ik onderzoek ging doen op nematoden (ik geloof dat ik ooit in mijn tweede bachelor de uitspraak gedaan heb dat nematoden de stomste beesten ooit zijn en ik die nooit meer wou zien), ik kon toch eindelijk mijn bijnaam "**Nematode Nele**" (enkel voor de leuke alliteratie, verder geen betekenis) waar maken!

Mijn eerste nematode uitpikken, ik herinner het mij nog goed, ging traag, zoooo traag. Als ik toen een snelle berekening had gemaakt over hoe lang ik er over gedaan had en dit vermenigvuldigd had met hoeveel nematoden ik nog ging uitpikken in mijn hele doctoraat, dan had ik waarschijnlijk niet meer verder gedaan omdat mijn pensioen dan wel al zeer dichtbij gekomen zou zijn!

En die nematoden: het zijn vreemde beestjes! Ik droomde ervan, zag ze als ik gewoon mijn ogen sloot en vooral: ik begon ze leuk te vinden. De cryptische soorten kregen al snel hun eigen "persoonlijkheid": Pm I was de gemakkelijke, Pm II de ambetante, Pm III de eigenwijze en Pm IV, ja wat met Pm IV eigenlijk? Daar ben ik nog niet helemaal aan uit. Wanneer ik nogmaals de opmerking kreeg "en wij betalen belastingen voor uw wormkes ofwa?" (cfr. Erik Herman) begon ik met veel plezier te vertellen waarom ik onderzoek deed op "die wormkes"!

Natuurlijk zou dit doctoraat niet geworden zijn wat het was zonder de steun, hulp, ervaring, … van vele andere mensen. De eerste die ik hierbij wil bedanken is mijn promotor **Tom**. Het was altijd fijn om samen experimenten te bedenken, resultaten te bespreken of te discussiëren over hoe onverwacht de effecten die we te zien kregen waren. Wat je me ook vooral geleerd hebt is om niet altijd te volgen in het aantal treatments die je per experiment er zou bij 'lappen'. Ik weet nog goed, bij mijn eerste experiment wou ik dit op vrijdagvoormiddag doen (en had in de namiddag practicum). Je zei me dat het zeker te doen zou zijn op een halve dag. Niet dus! Sinds dan reken ik minimum 1 dag om een experiment op te starten ☺. Tom, een echte oprechte bedankt recht uit mijn hart voor alle kansen die je me gegeven hebt en de menselijkheid die je toont in de wetenschap!

**Sofie**, mijn co-promotor, ik denk dat de meest hevige discussies, vloeken en zuchten, maar ook "hoera's"met jou uitgewisseld zijn! Het was altijd fijn om samen met jou naar een oplossing of verbetering te zoeken. Je vrolijkheid en openheid zorgden er steeds voor dat ik ook durfde om jou soms eens tegen te spreken ☺. Wat mis ik het om even snel langs je bureau te lopen en om dan meer dan een uur later met nog meer onzekerheden naar mijn bureau terug te keren! In ieder geval ben ik heel blij dat je het zo fijn hebt in je nieuwe job! En wanneer ik binnenkort naar Denemarken op reis vertrek, zal je niet-werk gerelateerd advies ook erg van pas komen!

Tom en Sofie, ik wil jullie allebei nog eens extra bedanken voor de warmte, begrip en interesse die jullie altijd toonden, niet alleen over wetenschap ook op persoonlijk vlak! Merci!

I also want to thank **all jury members** for the critical revision of my thesis. Their remarks and questions resulted in a better version of this PhD thesis. Thank you!

**Annelien**, zonder jou was ik nu nog altijd niet klaar met mijn eerste experiment, denk ik! Wat ongelofelijk fijn dat je altijd vol enthousiasme kon helpen aan mijn experimenten. Twee belangrijke dingen heb ik geleerd in die jaren samenwerken met jou: 1) laat nooit een wafeltje achter op je bureau (want Annelien eet het met plezier op) en 2) wij zouden heel slechte bureau-genootjes zijn aangezien we veel te veel kletsen en lachen!

Ook de andere personen van het **moleculair labo** wil ik zeker niet vergeten! Het was daar soms heel eenzaam, dus was het extra leuk als Pieter een muziekje oplegde of Sofie de laatste weetjes kwam vertellen! Jullie maakten CeMoFe een leuke plaats om te werken!

Aan mijn bureaugenootjes: **Ellen, Luana, Tania en Xiuqin.** Wat was het altijd leuk in onze 'tropische bureau'! Ik hou van drukte en veel gebabbel, ideaal dus! Het was altijd fijn om naar het werk te komen ☺ (ja, zelfs na 4,5 maand moederschapsverlof).

Maar ook alle anderen van het **labo** wil ik niet vergeten natuurlijk. Met sommigen van jullie ging ik op reis, met anderen deed ik experimenten, met nog anderen gaf ik practicum of gaven me een lift naar huis of had ik gewoon een fijne lunchpauze/koffiepauze. Sommige van jullie werden zelfs meer dan collega's. Om het met **Liesbet** haar woorden te zeggen "**als je samen iets doet en het is niet in werkverband, dan ben je vrienden!**". Wel Liesbet: je hebt gelijk.. en altijd toevallig op hetzelfde moment naar het toilet gaan, telt dat ook mee als vriendschap?

**Delphine:** mijn labomaatje! Wat is het hier anders zonder jou! We hebben veel leuke momenten gedeeld, ook veel minder leuke.. Maar het was altijd fijn dat je er was, onvoorwaardelijk! En ook heel fijn dat je er nog steeds bent, dat vriendschap ook over de landgrens blijft bestaan! Dikke knuffel! (en je weet hoeveel dat betekent voor mij!).

**Tim D. (en familie)**, we delen wel meerdere passies dan enkel mariene biologie! Ik wil je oprecht bedanken voor alle adviezen, uitdagingen en vakantiemomenten die we deelden. Het is heel fijn om te weten dat er iemand evenveel van onderwijs houdt dan mij! Ook bedankt om de kinderen eens uit te wisselen op spring school ☺.

**Tim T. en Niels D.:** wie had ooit verwacht dat ik van ons nog de eerste zou worden om te doctoreren? Bedankt om me in de eerste jaren van mijn doctoraat op te vangen als ik op de Ledeganck zat. De vele lunches en babbels tussendoor hebben echt deugd gedaan. En Tim, wat leuk dat jij nu gewoon bij ons werkt!

Als ik dit doctoraat nog een beetje dikker zou willen maken (maar daar zal Freija zeker niet mee akkoord gaan ;)) dan kan ik een heel lang lijstje maken van alle personen die ik wil bedanken omdat we samen iets **onderwijs-**gerelateerd gedaan hebben. Ik heb de kans en het geluk gehad om met enorm veel verschillende mensen practicum te mogen geven, naar Wimereux te gaan, Summer/Spring School te begeleiden, … ! Met zoveel verschillende personen kunnen samenwerken, maakt me niet alleen een beter wetenschapper, maar ook een beter persoon! Bedankt! Een speciale dankuwel gaat naar **Sandra, David en Ellen D.**: wat was het leuk jullie te leren kennen en met jullie een practicum voor de allereerste keer te laten doorgaan! De grootste merci hier is natuurlijk voor **Miranda:** wat een zalige madam ben jij! Alles is altijd op en top en bovendien kunnen we nog eens goed lachen ook!

Ik wil ook graag **alle studenten** bedanken. Mijn bachelorproef-, en masterproefstudenten, degene die meegingen op één of andere stage of zelfs gewoon degenen die in een practicum zaten. Jullie maakten van mij een beter wetenschapper! Jullie lieten me kritisch nadenken en jullie haalden het enthousiasme voor onderwijs in mij naar boven. Als ik thuis kwam na een practicum, mocht ik altijd horen dat ik glunderde. Want inderdaad, dat is wat ik zo fijn vind!

Al zeg ik het zelf, ik ben nog al goed in het werk van me afzetten als ik thuis kom. Dit is zeker omdat ik kon rekenen op een fantastische vriendengroep:

De **madammen** (van de VDS): supertof om eens een weekendje weg te kunnen gaan. Bij jullie is het altijd goed zot en kan ik al mijn energie kwijt. Leuk dat ik op restaurant al eens een dissectie van een mossel mag uitvoeren voor jullie!

Mijn **chiromaatjes en akabevriendjes** (ja ik ben een overloper) en de **Jossen**: ook al zien we elkaar niet altijd evenveel, wat is het leuk om gewoon met jullie het gevoel te hebben dat we toch nog 10 jaar jonger zijn!

**Jolien, Valerie en Evelyn:** bedankt om jullie zwangerschapsverlof zo mooi te timen met dat van mij (en Valerie: jij zelfs tweemaal ☺)! Ik kijk met heel veel plezier terug naar al die leuke wandelingetjes, pannenkoekjes en babymomentjes!

De leden van de **patatjesclub** (haha Lien, danku voor de naam): leuke etentjes (al dan niet wekelijks) en uitstapjes zijn het ideale recept voor een stressloos leven. Nu dat weekendje aan de Moezel nog!

**Erik, Jolien D, Roelandtjes** en alle personen die op een bepaalde manier te maken hebben met **Roemenië** (ook **de toneelspelers)**: bedankt! Multumesc! Prachtig, gek Roemenië..je veroverde mijn hart! Ik blijf zeker komen!

Mijn **treinmaatjes: Charlotte en Sara**. Wat is het leuk om het 'leed' van de NMBS te kunnen delen met jullie. En ook zo fijn om samen met jullie in dezelfde fase van het doctoraat te kunnen zitten (al nemen jullie dat misschien wel ietsje te letterlijk). Sara: ik beloof dat mijn fietsconditie snel weer beter zal zijn! Merci voor de leuke tijden op de trein! Ook aan **An-Sofie**: ons nieuw treinmaatje!

Mijn **broers, zus, schoonbroer, schoonzussen, kindjes en schoonfamilie**: wat een fantastisch leuk 'gezinnetje' hebben we toch! Ik kijk altijd uit naar het weekend om jullie te kunnen zien. Ik ben oprecht blij deel te kunnen maken van zo een gek, groot gezin!

**Moeke en vake**, zonder jullie zou ik er niet zijn, letterlijk maar ook figuurlijk. Moeke, ik herken steeds meer van mezelf in jou… maar daar kan ik alleen maar blij om zijn! Jullie gaven me de beste motivatie ooit om te studeren in mijn eerste bachelor 'als ge er niet door zijt, dan moet ge wat minder hobby's doen ze'. Boodschap begrepen! Bedankt voor alles!

De afgelopen 7 jaar zijn er niet alleen op werkvlak veel dingen gebeurd, maar ook op persoonlijk vlak. En lieve **Jens**, hier was jij de constante in! We gingen samenwonen, trouwen, kindje krijgen, huisje kopen en nog een kindje krijgen. Ik zou veel woorden kunnen schrijven, maar ik geef je er maar drie '**Take me away'**, dat zegt denk ik meer dan genoeg! Je maakt van ons huisje een thuisje, een plaats waar ik me veilig voel en niet aan het werk moet denken als ik daar geen zin in heb!

**Arwen en Lorin**: ook bij jullie schieten woorden tekort. Jullie maken elke dag een echt plezier en ook al werkten jullie niet goed mee in de laatste week voor het indienen van mijn thesis (Arwen: ziek, Lorin: niet meer doorslapen), jullie zijn fantastisch! Zoals Stef Bos het zou zingen (en die heeft altijd gelijk!): "**Ze noemen het liefde, ze noemen het alles, ze noemen het thuis, maar wat het ook is, jij bent het voor mij**!"

Bart DS maakte de vergelijking tussen doctoreren en bevallen. Ik wil daar graag wat dingen aan toevoegen: eerst en vooral bevallen is veel pijnlijker! Ik zou eerder de vergelijking maken tussen een kind opvoeden en doctoreren. Het gaat met vallen en opstaan, soms werken ze niet mee en moet je ze in de hoek zetten, maar meestal hou je ervan en ben je blij als ze iets bijgeleerd hebben. Mijn doctoraatskindje kan nu de wijde wereld in! Het gaat je goed!

Nele, maart 2016

# SAMENVATTING

Cryptische soorten zijn soorten die genetisch verschillend zijn maar als één soort geclassificeerd worden omwille van hun morfologische gelijkenissen. Cryptische soorten komen wereldwijd voor en zijn in nagenoeg alle taxa terug te vinden. Cryptische soorten gaan vaak sterk in interactie met elkaar, wat in overeenstemming is met Darwins competitietheorie: ze zijn zo gelijkaardig in morfologie en fysiologie dat een hoge graad van ecologische gelijkenissen verwacht wordt, waardoor competitie –de aanwezigheid van een bepaalde soort heeft een negatieve invloed op een andere soort- hoog zal zijn, en dit het samen voorkomen van soorten zal bemoeilijken, zeker op langere termijn en op kleine ruimtelijke schaal (= co-existentie). In de natuur komen vele cryptische soorten sympatrisch voor, wat er op wijst dat er mechanismen moeten bestaan om tot deze co-existentie te leiden. De meeste gaan uit van ecologische verschillen tussen de soorten en van het idee dat soorten zich, indien nodig, kunnen verplaatsen weg van hun geboorteplaats (= dispersie).

Het cryptisch nematodensoortencomplex van *Litoditis "marina"* (Sudhaus, 2011) is een ideaal model systeem om co-existentie van cryptische soorten te bestuderen. *L. "marina"* voedt zich met bacteriën en leeft op (veelal) rottende wieren in het intertidaal gebied. Er werden reeds 10 cryptische soorten teruggevonden binnen deze soort. Vier van deze soorten (Pm I, Pm II, Pm III en Pm IV) komen frequent voor langs de zuidwestkust en aanpalende estuaria van Nederland, en meestal worden twee of drie van deze cryptische soorten samen teruggevonden. Genetische verschillen tussen de cryptische soorten zijn vastgesteld in drie onafhankelijke loci en tot op heden is er geen reproductie tussen de soorten waargenomen. Bovendien zijn er geen diagnostische morfologische kenmerken waarmee de soorten eenduidig van elkaar kunnen onderscheiden worden; bij aanvang van dit doctoraatsonderzoek waren er ook geen ecologische verschillen tussen de soorten gekend. Dit alles maakt dat deze soorten kunnen beschouwd worden als echte cryptische soorten, waarvan het samen voorkomen de traditionele competitietheorie van Darwin in vraag stelt. In deze doctoraatsstudie onderzoeken we deze co-existentie, samen met de competitieve interacties die tussen de soorten kunnen voorkomen.

In het eerste experimentele hoofdstuk van het doctoraat onderzochten we of competitie tussen de cryptische soorten bestaat. In een labo-experiment met gesloten microkosmossen (zonder mogelijkheid tot dispersie) werd competitie tussen de vier cryptische soorten getest. Competitie bleek zeer algemeen en uitgesproken te zijn; twee van de vier soorten (Pm I en

Pm III) waren competitief superieur ten opzichte van de andere. Bovendien veranderde saliniteit de competitieve mogelijkheden van de soorten: interacties werden nog sterker bij een lage saliniteit en ofwel konden geen enkele van de vier soorten overleven ofwel werden de twee competitief inferieure soorten (Pm II en Pm IV) daar volledig weggeconcurreerd. Deze resultaten tonen aan dat co-existentie tussen de vier soorten bemoeilijkt wordt in een gesloten omgeving. Desondanks konden twee soorten (Pm I en Pm III) wel samen voorkomen. Dit resultaat, in combinatie met het sympatrisch voorkomen van de soorten in hun natuurlijk milieu (Derycke et al., 2006), wijst er op dat mechanismen die kunnen leiden naar co-existentie van deze cryptische soorten bestaan.

Niche differentiatie – verschillen in de ruimte bepaald door omgevingscondities en bronnen waarin soorten voorkomen en mee kunnen interageren– is één van de meest plausibele mechanismen voor co-existentie. In intertidale gebieden spelen saliniteit en temperatuur een belangrijke rol in de velddistributie van veel organismen. We onderzochten in een labo-experiment of levensgeschiedeniskenmerken (juveniele ontwikkelingstijd, fecunditeit en populatiegroei) en reproductieve strategieeën variëren tussen de soorten en/of in functie van deze twee abiotische factoren. Dit bleek inderdaad zo te zijn: Pm III vertoonde altijd de hoogste fecunditeit, ongeacht de abiotische condities. Temperatuur had een groter effect op de levensgeschiedeniskenmerken van alle soorten dan saliniteit. Pm II en Pm IV vertoonden de beste populatiegroei bij lage temperatuur, Pm III bij hogere temperatuur en Pm I vertoonde geen voorkeur. Deze verschillen zijn in overeenstemming met de velddistributie van de soorten aan de kust en de estuaria in de Noordzee (Derycke, et al., 2006). Deze resultaten wijzen er op dat verschillende voorkeuren tussen de cryptische soorten, ook al is het voor slechts één abiotische factor, reeds kunnen leiden tot niche differentiatie.
Het vorige experiment werd uitgevoerd bij verschillende temperaturen en saliniteiten, maar deze bleven constant over de tijd. Dit is niet in overeenstemming met de natuurlijke situatie waar zowel seizoenale als dagelijkse fluctuaties voorkomen. In een volgend experiment werden daarom de populatiegroei van de verschillende cryptische soorten en hun interacties getest bij dagelijkse fluctuerende temperaturen en vergeleken met een constante temperatuur. Fluctuerende temperaturen bleken geen effect te hebben op de fitness van de populaties, maar hadden een belangrijk effect op de interacties tussen de soorten. Afhankelijk van welke soorten gecombineerd werden, zorgde het temperatuurregime voor een verandering in de interacties (van mutualisme naar commensalisme) of in de sterkte van de interacties. Deze resultaten wijzen er op dat het zeer belangrijk is om het effect van

fluctuerende abiotische factoren op interacties te onderzoeken. Dit is van belang bij het voorspellen van het effect van klimaatverandering: klimaatvoorspellingen tonen aan dat de amplitude van dagelijkse temperatuurfluctuaties zullen verminderen. Bovendien is dit ook belangrijk voor co-existentie: ook al hebben abiotische condities geen effect op populatiegroei, zij kunnen dit wel hebben op de interacties. Co-existentie kan dus in een bepaalde omgeving niet mogelijk zijn, terwijl dit wel zo is als de abiotische condities veranderen.

Een ander mogelijk mechanisme van nichedifferentiatie is differentiatie in dieet. De bacteriële diversiteit van individuele nematoden van drie cryptische soorten werd onderzocht met behulp van Next Generation Sequencing. Dit experiment toonde aan dat het microbioom van de soorten zeer divers is en dat er een grote variabiliteit binnen de soorten bestaat. Bovendien werd het bestaan van soortspecifieke microbiomen bevestigd. Naast deze verschillen werd de bacteriële gemeenschap ook beinvloed door de aangeboden voedselbron en bleek dit soortafhankelijk te zijn: Pm III was meer selectief in zijn voedselopname dan Pm I.

De voorgaande drie hoofdstukken maken dus duidelijk dat er ecologische verschillen in abiotische voorkeuren en dieet bestaan tussen de cryptische soorten. Deze leiden tot nichedifferentiatie, wat kan leiden tot lokale co-existentie.

In het snel veranderende en kortstondige habitat waar *L. "marina"* voorkomt is dispersie belangrijk om te kunnen ontsnappen aan ongunstige condities. Bovendien is dispersie noodzakelijk in alle metagemeenschap paradigma's (regionale co-existentie). We onderzochten daarom ook of de cryptische soorten verschillen toonden in hun actieve dispersiemogelijkheden. Hiervoor maakten we gebruik van speciale dispersieplaatjes waar de nematoden de mogelijkheid kregen om zich van de ene locatie naar de andere te verplaatsen. Actieve dispersie van *L. "marina"* bleek veel voor te komen en was densiteits-, geslachts- en omgevingsafhankelijk. Bovendien bleken de dispersiemogelijkheden te verschillen tussen de soorten: Pm I was de traagste disperseerder en Pm III de snelste. Verschillen in dispersiemogelijkheden kunnen er voor zorgen dat soorten die competitief inferieur zijn als eerste in een lege patch aankomen en daar al een populatie kunnen opbouwen waardoor zij een voordeel hebben ten opzichte van later arriverende soorten, die competitief sterker zijn. Dispersieverschillen bepalen dus mee de gemeenschapscompositie en co-existentie van de soorten.

Competitie kan ook vermeden worden door dispersie. Dit werd getest aan de hand van een competitie-experiment met de vier cryptische soorten (vergelijkbaar met het eerste competitie-experiment), maar deze keer met de mogelijkheid om te disperseren. Daarbovenop onderzochten we of interspecifieke competitie, intraspecifieke competitie of voedselbeschikbaarheid de belangrijkste stimulans voor dispersie was. We vonden inderdaad dat co-existentie bevorderd kan worden door dispersie omdat competitie uitgesteld of vermeden kan worden. We konden ook aantonen dat de stimulans om te disperseren soortafhankelijk is: Pm III en Pm IV disperseerden wanneer de densiteit (ongeacht de soort) te hoog werd. Pm II disperseerde altijd op hetzelfde moment, ongeacht de densiteit, en Pm I disperseerde sneller wanneer interspecifieke interacties aanwezig waren.

Al deze ecologische verschillen tussen de cryptische soorten kunnen potentieel ook leiden tot functionele verschillen. We onderzochten daarom of de verschillende cryptische soorten een verschillende rol hebben in het functioneren van een ecosysteem. We deden dit door het effect van de soortidentiteit op de decompositie van organisch materiaal (algen) te bekijken, alsook het effect op de activiteit van twee sleutelenzymen, die mee instaan voor de afbraak van het algenmateriaal. Bovendien werd er ook getest of interspecifieke interacties een invloed hadden op het decompositieproces. Verschillen tussen de soorten werden teruggevonden. Pm IV is vermoedelijk belangrijker in het stimuleren van het decompositieproces, terwijl Pm II geen enkel duidelijk effect vertoont. De interspecifieke interacties die gevonden werden zijn vergelijkbaar met deze van het eerste competitie-experiment: Pm I werd zeer dominant. Toch bleek dat in deze culturen het effect op decompositie verschilde van de culturen waar alleen Pm I voorkwam. Interspecifieke interacties kunnen dus leiden tot een verschillend effect op decompositie. Bij gevolg zal dus elke cryptische soort potentieel belangrijk zijn voor het functioneren van ecosystemen en kan haar belang geheel of gedeeltelijk gemodificeerd worden door de aanwezigheid van andere cryptische soorten.

We kunnen samenvatten dat interacties tussen de cryptische soorten veelvuldig voorkomen. Hoewel we in het eerste competitie-experiment met de gesloten microkosmossen een duidelijke hiërarchie van competitieve soorten waarnamen, was het onmogelijk om één competitief superieure soort aan te duiden wanneer we alle experimenten in rekening brengen. Er bestaat een competitief intransitief netwerk, waarbij de soorten niet eenvoudig kunnen gerangschikt worden naargelang hun competitieve mogelijkheden omdat een

bepaalde soort wel competitief dominant kan zijn over een andere soort, maar tegelijkertijd ook competitief inferieur vergeleken met nog een andere soort. De cryptische soorten verschillen in meer aspecten dan verwacht: verschillen in levensgeschiedeniskenmerken, dieet, competitieve mogelijkheden, dispersiestrategieën en hun rol in het functioneren van een ecosysteem komen voor. Deze verschillen resulteren in nichedifferentiatie en kunnen, gecombineerd met dispersie, belangrijk zijn voor co-existentie. Bovendien wijst de verschillende rol die de soorten hebben in het functioneren van een ecosysteem er op dat het zeer belangrijk is om rekening te houden met cryptische diversiteit bij conservatiebiologie.

# SUMMARY

Cryptic species are species which are genetically distinct, but have been classified as a single species because of their morphological similarity. Cryptic species have been found worldwide and in many different taxa. Strong interspecific interactions are common within cryptic species complexes. According to Darwin's competition theory this may be explained by the fact that cryptic species are so similar in their morphology and physiology that a high degree of ecological similarity is present. As a result, competition - the presence of one species influence the occurrence of another in a negative way- is fierce and co-occurrence of the species on a larger temporal scale and small spatial scale (= coexistence) is hard to achieve. Nevertheless, many cryptic species complexes have sympatric distributions, so some mechanisms which facilitate coexistence have to exist. Most mechanisms to achieve coexistence rely on the assumptions that some ecological differences between the species are present (i.e. niche differentiation) and that species are able to disperse from their natal habitat if necessary.

The cryptic nematode species complex of *Litoditis "marina"* (Sudhaus, 2011) is an ideal model to study coexistence of cryptic species. *L. "marina"* is a bacterial feeder and lives on decaying macroalgae in an intertidal environment. At least ten cryptic species have been found within this morphospecies complex, and four of them (Pm I, Pm II, Pm III and Pm IV) occur along the south-western coast and estuaries of The Netherlands. It is common to find two or three of these cryptic species co-occurring. Consistent molecular divergences between these species have been found at three independent loci and the species are reproductively isolated. No diagnostic morphological characters are present and ecological diversification had not been documented at the start of this PhD. As such, these species challenge the traditional competition theory of Darwin. In this PhD thesis we investigated the coexistence of these four cryptic species of *L. "marina"* and the competitive interactions between them.

First of all, we investigated whether competition exists between these co-occuring species. In a laboratory experiment, competition between the four cryptic species was tested in a closed environment without dispersal possibilities. Competition between the species was found to be common and pronounced, with two species (Pm I and Pm III) being competitively superior over the other two. Moreover, changing the abiotic environment (*in*

*casu* salinity) modified the competitive abilities of the species. The interactions became even stronger at lower salinity, resulting in the complete exclusion of all four species in some plates and the complete exclusion of the two competively inferior species (Pm II and Pm IV) in others. These results demonstrate that coexistence between the four species in a closed environment is difficult to achieve. Nevertheless, two of the four species were still present in high abundances at the end of the experiment and there is also evidence of co-occurrence of the cryptic species in the field, so some mechanisms leading to their coexistence have to exist.

Niche differentiation – differences in the multi-dimensional space of resources in which an organism can respond and alter– is one of the most common ways to achieve coexistence. In intertidal marine environments salinity and temperature play an important role in determining species distributions.We performed a lab experiment to investigate whether cryptic species differ in their life histories (juvenile development time, instantaneous fecundity and population development) and in their response to these abiotic conditions. Pm III had a higher instantaneous fecundity than the other three species regardless abiotic conditions. Temperature and salinity affected the life history characters and the reproductive strategy of some of the cryptic species, with temperature clearly having a stronger effect than salinity. Pm II and Pm IV performed better at lower temperatures, Pm III at higher temperatures, and Pm I did not show any temperature preference. These different temperature preferences nicely correlate with the respective seasonal field distribution patterns of these cryptic species along the south-eastern coast of the North Sea and adjacent estuaries (Derycke, et al., 2006). This suggests that different preferences of the cryptic species for even a single abiotic factor may already result in niche differentiation.

The previous experiment was performed at different but constant temperatures and salinities. In nature, these abiotic conditions are not constant but fluctuate at both a seasonal and daily basis. Therefore, in a next experiment, we compared population performance of individual cryptic species and the outcome of their interspecific interactions under a temperature regime with daily fluctuations vs a constant temperature regime. A fluctuating temperature regime had minor or no effects compared to a constant temperature on the population performance of the individual cryptic species, but did have a substantial effect on the interactions between them. Dependent on the combination of the species, temperature regime also changed the strength of the interspecific interactions or the type of interaction (from a sort of mutualism to commensalism). It is thus important to incorporate the effect of abiotic fluctuations on

interspecific interactions, for instance to predict the effect of climate change on biodiversity, as climate change models predict decreasing amplitudes of daily temperature fluctuations. Moreover, these results indicate that even if abiotic conditions do not have an effect on population development, interactions may be affected. Coexistence may thus not be present in one particular environment, but can then be achieved when abiotic conditions change.

Another potential driver of niche differentiation is differential resource use among the different cryptic species. Next Generation Sequencing on the microbial diversity in single nematode individuals from three cryptic *L. "marina"* species was conducted to investigate possible differences in food resource use of the species. Our results showed that the bacterial communities are highly diverse and show pronounced intraspecific variability. Moreover, we confirmed the existence of species-specific microbiomes. In addition to differences in 'gut microflora' between cryptic species, an experiment with two different bacterial inocula as food source substantially affected the nematode microbiomes, illustrating different feeding behaviour between the cryptic species, with Pm III being a more selective feeder than Pm I.

Based on the previous, it is clear that ecological differences in abiotic preferences and resource use exist between the cryptic species. This niche differentiation can potentially provide an important mechanism for local coexistence of these closely related species.

In the rapidly changing and ephemeral habitat where *L. "marina"* lives, dispersal is extremely important to avoid unfavourable conditions and is required to achieve regional coexistence. We investigated whether individual cryptic species show differences in their active dispersal abilities. We used specially designed dispersal plates, where nematodes were able to move from one 'patch' to another. Active dispersal in *L. "marina"* was common, density-dependent and gender- and environment-specific. Moreover, species-specific differences existed: Pm I was the slowest disperser and Pm III the fastest. Dispersal differences can lead to competitively inferior species arriving first at an empty patch and starting a new population, which can be beneficial over (more competitively superior) species arriving later. As a result differential dispersal can shape community composition and coexistence.

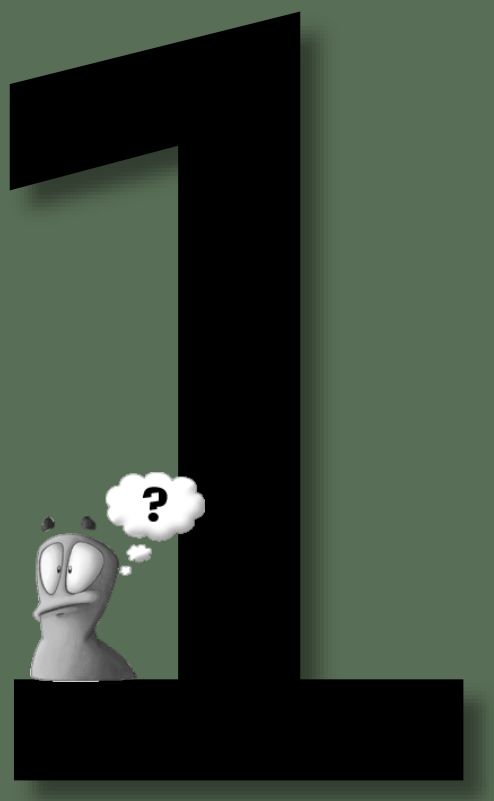
Competition can also be avoided through dispersal. To test this, we conducted a competition experiment like the first one with the four cryptic species together but now offering opportunities for dispersal rather than working in a closed microcosm without dispersal. Moreover, we tested whether interspecific competition, intraspecific competition or food

availability was the main driver of dispersal. We found that co-occurrence can be facilitated because competition is postponed or avoided by dispersal. All four species were able to co-occur in fairly stable abundances. Moreover, we showed that the trigger for dispersal is species-specific: density *per se* was the main driver for dispersal in Pm III and Pm IV. Dispersal of Pm II always started at the same timing irrespective of nematode density, whereas interspecific interactions were the main trigger of dispersal in Pm I.

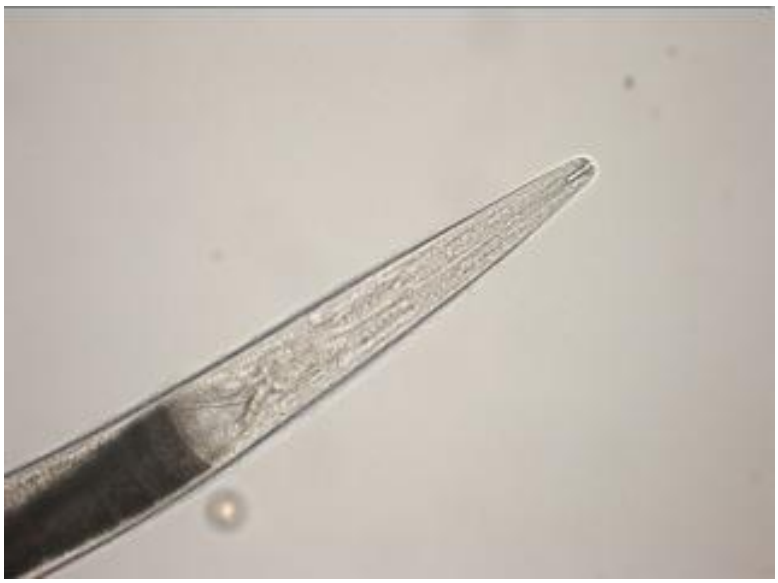
Ecological differences between the cryptic species could potentially translate in functional differences. *L. "marina"* can substantially impact the decomposition process of algal litter, and cryptic species may have differential effects. We therefore investigated whether different cryptic species have different functional roles in ecosystem functioning. Moreover, we tested if interspecific interactions among the four cryptic species influence the decomposition process. We tested this by studying the effect of the different cryptic species in single-species treatments and in a mix of species on the loss of organic matter from decomposing algae and on the activity of two key extracellular enzymes involved in the degradation of this phytodetritus. We demonstrated that species-specific effects on the decomposition process exist. Pm IV may be more important in accelerating the decomposition process compared with the other species and Pm II may not have any stimulatory effect at all. Moreover, interspecific interactions were comparable with the ones found in the first competition experiment: combining the four cryptic species resulted in high competition, with Pm I as dominant species, but without complete exclusion of other species. Nevertheless, the effects on the decomposition process was different in these combined cultures compared with cultures where Pm I occurred alone. As a result, each cryptic species may play a different role in ecosystem functioning.

We can conclude that interspecific interactions are prominent between the cryptic species of *L. "marina"*. Despite the clear competitive hierarchy found in closed microcosms in the first competition experiment, it was impossible to detect one ultimate competitively superior species across all experiments. Instead, we found evidence for the presence of a competitively intransitive network, in which species' abilities cannot be ranked in a hierarchy, because a species that is competitively superior over one species, may be competitively inferior compared to another species. In addition, it is clear that the cryptic species differ in life-history traits, abiotic preferences, feeding ecology, competitive abilities, dispersal strategies and ecosystem functioning. These differences result in niche differentiation, and combined with differences in dispersal abilities may lead to coexistence.

Finally, the differences in ecosystem functioning between the species emphasize the importance of including cryptic species in conservation management.



# CHAPTER I:



# GENERAL INTRODUCTION, AIMS AND OUTLINE OF THE THESIS

## Unravelling coexistence of cryptic species

The title of my PhD 'coexistence of cryptic species' immediately raises three important questions: (1) what is a **species**, (2) what does **cryptic** mean, and (3) what is **coexistence**? The general introduction of this work will first focus on these three aspects, followed by an overview of different paradigms to achieve **coexistence**, applied to cryptic species, and a short explanation why cryptic diversity may be important. I will conclude this by integrating this information with the outline of my PhD thesis.

## The species concept

Studying nature already fascinated humans from the early cultures (Indian, Egyptian, Mesopotamian, Chinese) in which passing on knowledge about plants and animals was extremely important to increase their chances of survival (Magner, 2002). The Sushruta Samhita, for instance, was an Indian collection of medical information and described more than 700 medicinal plants, 64 medical preparations from mineral sources, and 57 preparations based on animal sources (Bhishagratna, 1963). Still, it must have been Aristotle who was one of the first to really study the diversity of life and classify animals into "categories" (Singer, 1931; Mayr, 1982). He arranged creatures in a 'scala naturae' (Fig. 1): a graded scale of perfection rising from plants to human beings (Granger, 1985). Nevertheless, the first real definition of 'a species' was only formulated by John Ray in 1686: species were 'units' distinguished by always producing the same form, but considerable variation was possible (Wilkins, 2006). In 1859 Charles Darwin started the real discussion about the term species. "No one definition (of species) has as yet satisfied all naturalists…", he wrote "… yet every naturalist knows vaguely what he means when he speaks of a species. Generally the term includes the unknown element of a distinct act of creation" (Darwin, 1859). Combined with Mendel's theory of inheritance, biologists started to think about the exact definition of a species. Ernst Mayr was the first to define species with the **biological species concept** (Mayr, 1942), in which species are reproductively isolated. As a consequence, different species are necessarily reproductively isolated and are representing separate evolutionary lineages. Nevertheless, this definition has some pitfalls: distinguishing potential reproductive barriers can be difficult, and the biological species concept cannot be straightforwardly applied to asexual organisms and allopatric populations. Since then, the exact definition of the word species has been discussed for a long time and is still the subject of different opinions. More than twenty species concepts remain in circulation (Mayden,

1997). Most older species descriptions rely on the typological species concept (Bickford, et al., 2007): organisms are classified in the same species if they appear identical by **morphological criteria**. Such a morphological classification mostly leads to the fact that populations and not specimens are being identified as species (Agapow, et al., 2004). In the past, researchers have accidentally classified male and female individuals of the same (biological) species into separate species, or have lumped two species in one when they are morphologically very similar. For this reason, a genetic definition of the term species may be more meaningful because it is related to the evolutionary processes that give rise to the groups being classified. The **phylogenetic species concept** considers a species to be a group of organisms that have descended from a common ancestor (Cracraft, 1983). This concept has also some pitfalls: what with neutral mutations? Or what level of divergence is needed to constitute a species (De Queiroz, 2007)? For this reason, nowadays, **integrative taxonomists** look for changes in more than one type of characteristic of an organism and try to incorporate independent data of molecules, morphology or mating signals. This is mostly deemed to be good evidence for separating species (Bickford, et al., 2007). In this PhD, we will use a combination of the biological species concept (species do not interbreed) and the phylogenetic species concept (species show genetic differences).

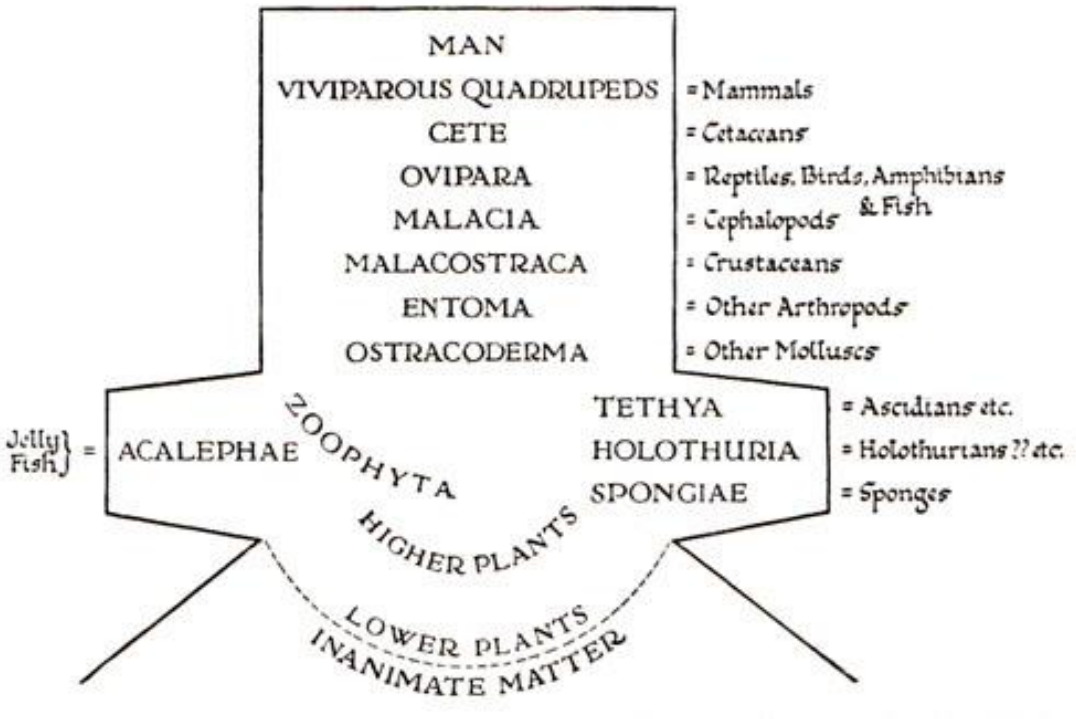



Figure 1: The Scala Naturae or 'great chain of being' according to the descriptions of Aristotle (Singer, 1931).

## Cryptic diversity

When two or more **genetically distinct species are classified as a single species** because of their **morphological similarity**, they are called cryptic species (Knowlton, 1993). Cryptic species may result from speciation that is so recent that morphological traits have not yet evolved. Nevertheless, evidence for more ancient speciation (millions of years ago) was also found (Bickford, et al., 2007), without the presence of any obvious morphological differences. Three main reasons may explain why such species do not differ in morphology : (1) **speciation is not always accompanied by morphological change**: cryptic species may be differentiated by nonvisual mating signals or behavioural differences (Bickford, et al., 2007), or be under selection that promotes morphological stasis (Gómez, et al., 2002). (2) Species look morphologically similar at first sight, but **once they are studied in more detail, diagnostic characters can be found** (Pfenninger & Schwenk, 2007). Some suggest to call these species 'pseudo-cryptic', but in general, the definition of cryptic species states that species are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable (Bickford, et al., 2007). And (3) the existence of **convergent evolution**: independent evolution of similar features in species of different lineages, which creates analogous structures that have similar form or function (Henry, et al., 1999; Jones & Holderied, 2007). Evidence of **recent cryptic speciation** was found for two species of coccolithophores (Sáez & Lozano, 2005). Another example are two recently diverged cryptic species of sea urchins (Landry, et al., 2003), which hitherto only appear to differ in sperm morphology. Most cryptic species diverged, however, **millions of years ago** (e.g. fish, copepods, amphipods, algae,... (Colborn, et al., 2001; Rocha-Olivares, et al., 2001; Lefébure, et al., 2006; Šlapeta, et al., 2006)).

## Sympatric occurrence of cryptic species

Although allopatric distributions – geographically separated ranges – of cryptic species do exist (e.g. Solé-Cava, et al., 1991; Chang, et al., 2008), most cryptic species complexes have **sympatric distributions**: they occupy the same or overlapping geographic areas (e.g. Pinto, et al., 1986; Knowlton, 1993; Trewick, 1998; Mayer & Von Helversen, 2001; Ortells, et al., 2003; Zhang, et al., 2004; Derycke, et al., 2006; Stuart, et al., 2006; Amato, et al., 2007; Wellborn & Cothran, 2007; Montero-Pau, et al., 2011). Sympatric distributions may be the result of sympatric speciation, where intraspecific competition is strong enough to induce disruptive selection (Bürger, et al., 2006; Svanbäck & Bolnick, 2007) and/or adaptations leading to speciation, or of allopatric speciation followed by range expansion. The first mechanism has been proposed to explain the sympatric occurrence of cryptic species of damselflies (McPeek & Brown, 2000), the second for amphipods and nematodes (Witt & Hebert, 2000; Derycke, et al., 2008b).

Interactions between sympatric cryptic species are common: for instance, some cryptic rotifer species showed strong **interspecific competition** and even competitive exclusion (Ciros-Pérez, et al., 2001). This may be explained by the fact that cryptic species are so similar in their morphology and physiology that a high degree of ecological similarity is expected (Leibold & McPeek, 2006), which may lead to strong competition - the presence of one species influence the occurrence of another in a negative way - between them (Darwin, 1859). Two main types of competition may exist between cryptic species: (1) **exploitation** competition and (2) **interference** competition. The first is also called resource competition: organisms will compete for food, space or other necessary resources. This has, for instance, been observed for cryptic species of planktonic algae (Tilman, 1977). The competitively inferior species is not able to find enough resources, because the superior one(s) deplete(s) the resource faster or better. In interference competition, one species interferes with the ability of another species to obtain resources (Schoener, 1974), for instance through aggressive behaviour. A more competitive species may show competitive behaviour combined with predation and directly predate on eggs or juveniles of the competing species (intraguild predation; Case & Gilpin, 1974). Interference competition may also be caused by chemical repulsion as already proposed as one of the possible mechanisms for inhibition in marine harpacticoid copepods (Chandler & Fleeger, 1987): mucus tubes of one species inhibited the presence of a second species. Besides competition, other interactions between species may also occur such as facilitative interactions - the

presence of one species improves the occurrence of another - which may enhance coexistence. Nevertheless, due to the expected high competition between very similar species, long-term sympatric occurrence on a small spatial scale– further referred to as **coexistence** – of cryptic species **may be hard to achieve**.

**Coexistence** can be studied at two spatial scales: local and regional and is mostly evaluated as populations reaching an equilibrium state. If interspecific interactions, however, are disrupted under certain conditions (for instance in highly fluctuating environments), such an equilibrium state may be hard to achieve and non-equilibrium coexistence may exist (Pickett, 1980). At the **local scale**, no neighbouring patches are taken into account, and coexistence can only be achieved when there is niche differentiation and heterogeneity (Mouquet & Loreau, 2002). This essentially corresponds to a closed environment without dispersal opportunities, in which permanent coexistence of closely related species can only exist if intraspecific competition is equal to or higher than interspecific competition (Chesson, 2000a). Coexistence at the **regional or metacommunity scale** – a set of interacting communities which are linked by dispersal (MacArthur & Wilson, 1967) – can still occur. In this case, coexistence is a result of regional processes, such as immigration and extinction (Mouquet & Loreau, 2002). In this way, coexistence may be absent at the local scale, but may exist at the metacommunity level (Hanski, 1999). Hence, while species occur together at the regional level, they may be temporally or spatially segregated at the local level (mostly referred to as 'co-occurrence' instead of 'coexistence'). These different ways to achieve local coexistence (niche differentiation) and regional coexistence (metacommunity pardigms) are described in more detail below and are illustrated in Fig. 2. Which paradigm(s) explain(s) the sympatric occurrence of cryptic species is often difficult to establish, the more so since the different paradigms are not necessarily discrete and separate but may rather represent a continuum (Leibold & McPeek, 2006).

**Local coexistence**

*A. Niche differentiation*

The niche is a multi-dimensional space of resources in which an organism can respond and interact (Hutchinson & MacArthur, 1959; Hughes, et al., 2008). Coexistence of very similar species may be facilitated by some distinct niche differences (Zhang, et al., 2004). In most cryptic species complexes, ecological and functional differences are largely unknown, but subtle morphological, geographical and other differences are often present (Bickford, et al.,

2007). These may form a basis for niche differentiation which can be temporal or morphological (see further). In **temporal niche partitioning**, for instance, two species feeding on the same resource will minimize competition by utilizing the same resource at different times. This can be achieved at different time scales, from for instance day-night partitioning (Kronfeld-Schor & Dayan, 1999) to a seasonal scale, where different species reach peak abundances at different times of the year (Schoener, 1974; Lawler & Morin, 1993). In addition to **resource differentiation**, species can also exhibit differences in abiotic preferences, and coexistence may particularly be achieved in environments with fluctuating dynamics. The salinity tolerance ranges of cryptic rotifer species, for instance, overlapped, which affected their relative fitness (Gabaldón, et al., 2015). This has implications for competition and as a consequence for the coexistence of the species (Leibold & McPeek, 2006). A competing species needs to be able to recover from low densities in a fluctuating environment. Three conditions should be met to achieve this (Chesson, 2000b): 1) differential responses of the competing species to a **fluctuating environment**; 2) a **relationship between environment and competition**: some species will show better competitive abilities in a certain environment, but if the environment changes, another species will become more competitive ("heterogeneous competitive environment", Amarasekare, 2003; Montero-Pau, et al., 2011), and 3) **a 'stage' buffered from competition**, which may be a diapausing stage (Warner & Chesson, 1985) within that population or individuals from another patch that can disperse into this population (spatial storage effect, see further).

Another mechanism to avoid competition is selection for different morphologies. For instance, different bumblebee species show adaptations of the proboscis to other food sources (Goulson & Darvill, 2004). In this scenario, **character displacement** is the main mechanism behind coexistence (Abrams, 1986). In nematodes, polyphenism of feeding structures in response to different environmental conditions within the nematode *Pristionchus pacificus* may be an important step towards phenotypic evolution and (cryptic) speciation (Kiontke & Fitch, 2010). Resource diversification and differential abiotic preferences may not only be important mechanisms for local coexistence of species, but also for regional coexistence.

**Regional coexistence**

*B. Patch-dynamics: colonization-competition trade-off*

The **competition-colonization trade-off** has long been considered an important mechanism explaining species coexistence in spatially structured environments (Cadotte, et al., 2006). This mechanism relies on the principle that **species that are very good colonizers** (a combination of good dispersal capacities and a high fecundity) **may be poor competitors** and vice versa (Hastings, 1980). The underlying assumptions for this trade-off mechanism are that (a) all patches are connected by **dispersal**, (b) patches do not differ in their suitability for the competing species ('homogeneous environment') (Leibold, et al., 2004), and (c) there is a **competitive hierarchy** in a competitively homogeneous environment: competitive abilities do not change in response to environmental changes (Amarasekare, 2004; Calcagno, et al., 2006). Poor competitors will disperse to empty patches, where the stronger competitor will not (be able to) disperse to. As a result, species composition will vary in patches depending on whether species effectively colonize or outcompete each other (Winegardner, et al., 2012). Many experimental studies have failed to detect evidence for the dispersal-competition trade-off (reviewed in Cadotte, et al., 2006), because **spatial heterogeneity may overwhelm** the trade-offs. Due to this, it is often suggested that this trade-off can only account for the coexistence when there is an additional driver of coexistence (Amarasekare, 2003). Nevertheless, for instance in rotifer species (Cadotte, et al., 2006), competition-colonization trade-offs may be a very important mechanism for coexistence.

*C. Spatial storage (species sorting)*

Niche differentiation – such as differences in abiotic preferences (see above) – may also be important at the regional scale. In a **competitively heterogeneous environment**, where competitive abilities can vary in response to environmental changes (Crombie, 1947; Dunson & Travis, 1991), **competitively inferior species** (inferior in that specific place and at that particular time) may persist because they are **temporarily favoured by specific conditions** (Begon, et al., 1996). If the conditions change, other species may be favoured and will be able to dominate. This leads to species sorting: species will occupy suitable sites along environmental gradients and as a consequence, community composition may change by environmental perturbations. Dispersal is important to maintain species with a negative growth rate in a specific site at a specific time, and may buffer the species from extinction during those periods or in those places where their competitive abilities are impaired. Nevertheless, **dispersal is never sufficient to alter their distribution**. Priority effects

caused by dispersal limitation can lead to different stable communities composed of essentially the same species (Winegardner, et al., 2012): species that start a new population early in a certain patch, for instance because they are the first to arrive there, may have a greater chance of becoming and remaining dominant than later arriving species if they rapidly reproduce and are able to adapt to the new environment (Harper, 1961; De Meester, et al., 2002). The importance of **priority effects** to explain community structure has already been demonstrated in a wide range of taxa (Shulman, et al., 1983; Alford & Wilbur, 1985; Blaustein & Margalit, 1996; van de Voorde, et al., 2011). Not only differences in abiotic preferences may lead to species sorting, but also **biotic differences**, such as a differential vulnerability to predation, may provide important mechanisms behind species sorting. In this case, changes in the species composition are correlated with predation intensity and/or predator identity (Black II & Hairston, 1988; Garcia & Mittelbach, 2008). In contrast with the assumption of a homogeneous environment in the competition-colonization trade-off, **environmental fluctuations** are necessary to achieve coexistence in the spatial storage theory, and these environmental shifts need to occur before competitive exclusion can occur (Hebert & Crease, 1980).

Recently, there is even evidence for processes similar to the spatial storage concept but without species-specific differences in life history or competitive abilities. Coexistence may arise **from the dispersal process itself,** without spatial heterogeneity or biological trade-offs. This may be quite common in marine environments, in which dispersal of many organisms is mostly modulated by the physical environment and hence largely passive (Aiken & Navarrete, 2014). Connectivity of marine populations will vary over a range of space and time scales due to, for instance, local ocean currents. Nearshore marine species mostly have a short spawning period and the distance larvae can disperse depends on the characteristics of the flow, which may vary over time. This may result in the ability of competitively inferior species to survive, and differences in community composition may be achieved if species-specific differences in dispersal exist, such as the timing of spawning, or the buoyancy of larvae. Coexistence of reef fishes, for instance, may be possible when interspecific differences in dispersal abilities exist and if reef patches are distributed at irregular distances. Species will be able to disperse over different distances, depending on the oceanographic conditions (Bode, et al., 2011), and this will shape community composition. Freshwater invertebrate community composition will also be partly the result of a species sorting system due to temporal changes in wind speed that regulate their passive

dispersal. Some differences in egg morphology may lead to differences in dispersal distance between the species (Vanschoenwinkel, et al., 2007).

*D. Mass-effect paradigm*

The mass-effect paradigm acts in an environment where **different patches have different conditions at a given time**. Dispersal connects the different patches and creates **source-sink dynamics** (Leibold, et al., 2004). Populations may continue to persist in sink populations – where their net reproductive rate is less than their replacement – because of immigration from source populations, which live in a higher quality habitat that allows the populations to increase. The sink habitat would not be able to support a population on its own (Winegardner, et al., 2012). **High dispersal** is important in this paradigm as it ensures a constant supply of new colonizers to the sink habitats. The mass-effect paradigm implies that some habitat patches may be more important for the long-term survival of a population (Pulliam, 1988) than others and that species differ in some aspect: a source population for one species can be a sink population for the other. In contrast with the spatial storage paradigm, **temporal fluctuations are less important** for the coexistence of the species.

*E. Neutral dynamics*

In contrast to the four drivers of coexistence discussed above, the **neutral dynamics theory does not require species to be different** in order to coexist (Hubbell, 2006). If differences between species are completely unrelated to traits that influence fitness or demography, we can consider species as equivalent (Leibold & McPeek, 2006). In neutral theory (Hubbell, 2001), the presence of species is entirely and only driven by chance events, such as **random dispersal, ecological drift (demographic stochasticity) and mutation-order speciation** (Hubbell, 2001; Leibold & McPeek, 2006; Schluter, 2009). Nevertheless, complex ecological interactions, e.g. competition, can still exist among individuals or populations of an ecological community as long as all the individuals obey the same rules (Hubbell, 2005) and none of the species will be favoured in the end. Because species traits do not matter for fitness, species become so identical that **intraspecific competition is equivalent to interspecific competition** (Leibold & McPeek, 2006; Leibold, 2008); the outcome of these interactions, then, only depends on neutral processes. Notwithstanding the importance of niche differentiation to explain population and community compositions, neutral dynamics are sometimes surprisingly accurate in explaining patterns of coexistence (McPeek & Brown, 2000; Siepielski, et al., 2010; Langenheder & Székely, 2011).

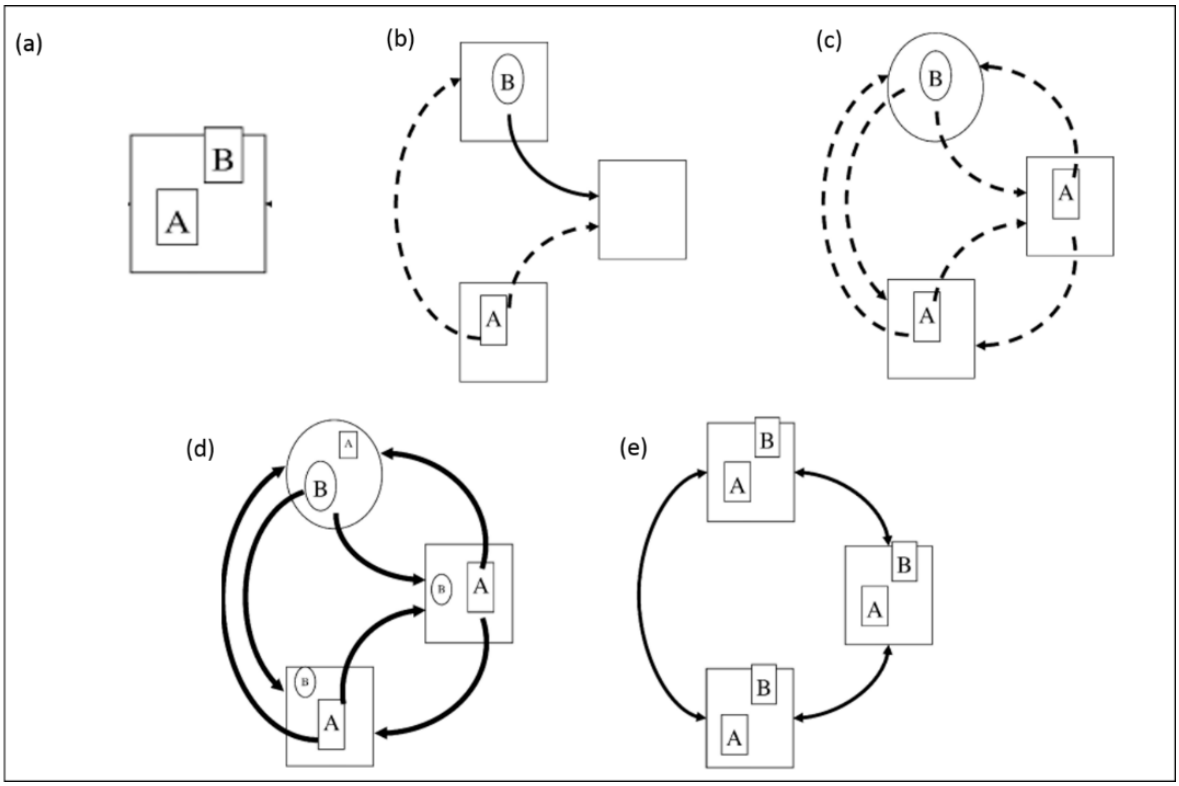



Figure 2: Schematic representation of paradigms of metacommunity theory (adapted from Leibold, et al. 2004) for two species represented by populations A and B. Arrows connect donor (= source) populations with potential colonization sites, shown as large boxes or ovals. If both boxes and ovals are present, the environment is heterogeneous. Solid arrows indicate higher dispersal than dashed arrows and either unidirectional movement (single-headed arrows) or bidirectional movement (double-headed arrows). The degree to which a species is the dominant competitor in a site is shown by the matching of the smaller box or oval (denoting its habitat type niche) with the site symbol (when the two coincide, this dominant competitor outcompetes or excludes the weaker competitor). The paradigms illustrated are (a) niche differentiation in a closed environment, (b) competition-colonization trade-off, (c) spatial storage, (d) mass-effects and (e) neutral dynamics. In all paradigms except (a), competition between the species occurs. In all paradigms except (e), some kind of niche or dispersal differentiation is necessary to achieve coexistence. In (c) and (d) a competitively heterogeneous environment exists.

## (Why) does cryptic diversity matter?

Cryptic species have been found **worldwide** in many different taxa (Pfenninger & Schwenk, 2007) and are also being discovered **within well studied taxa and geographic regions** (Bowen, et al., 1993; Mayer & Von Helversen, 2001; Rohland, et al., 2010). Detecting cryptic species is in the first place important for biologists studying evolution (see above) and ecology (Hebert, et al., 2004). Failing to recognize cryptic species may, however, also lead to an **underestimation of diversity** and an overestimation of the geographical ranges of individual species (Prada, et al., 2014), which may have important consequences for our understanding of how species adapt to changing conditions (Leavitt, et al., 2013), and for implementation of efficient biological control systems, for health issues and for the identification of invasive species (Bickford, et al., 2007). It may also have repercussions for **conservation biology**.

Conservation biology addresses the biology of species, communities, and ecosystems that are disturbed, either directly or indirectly, by humans and its main goal is to preserve biodiversity (Soulé, 1985). Ecosystems are experiencing accelerating loss of populations and species (Solan, et al., 2004; Díaz, et al., 2006; Worm, et al., 2006). The discovery of cryptic species also reveals a potentially significant underestimation of the true scale of this biodiversity loss (Bálint, et al., 2011). In aquatic insects, for instance, different cryptic species were discovered in a species that was supposed to have a very broad geographical distribution. Predicted future range contractions – as a result of global change – will thus likely be accompanied by **severe losses of cryptic species** (Bickford, et al., 2007). This may in turn have **consequences for ecosystem services**.

Ecosystem services are defined as benefits that mankind obtains from ecosystems and consist of supporting services (nutrient recycling, primary production, soil formation), provision services (food, water, raw materials, medicinal resources, …), regulating services (climate regulation, water purification, waste decomposition, pest an disease control, ….) and cultural services (recreational, science, …) (Millennium Ecosystem Assessment, 2005). Ecosystem services such as resource gains, recovery potential, ecosystem stability and water quality have already been shown to decrease with declining diversity (Loreau, et al., 2001; Covich, et al., 2004; Worm, et al., 2006). The role of cryptic species in this is largely unknown. On the one hand each cryptic species may be individually important for ecosystem services. Very similar species of decapods, for instance, have differential effects on energy flows and nutrient cycling (Covich, et al., 1999; Norling, et al., 2007). On the other hand,

plant vegetation studies have shown that the role of one species can be easily taken over by other functionally similar species (Burrows, 1990).

Different cryptic species may also require different **conservation strategies** (Bickford, et al., 2007). Knowledge of the geographical patterns of diversity is necessary for identifying biodiversity hotspots, which may consequently receive a special conservation status (Meegaskumbura, et al., 2002). In addition, endangered species might be complexes of multiple cryptic species, that are even rarer or have more contracted ranges than previously thought (Pfenninger & Schwenk, 2007); such insights may have repercussions for their conservation status (Funk, et al., 2011), as already demonstrated in frogs and lemurs (Bowen, et al., 1993; Ravaoarimanana, et al., 2004).

**Invasive species** endanger local biodiversity and should be controlled (Clavero & García-Berthou, 2005). Cryptic species may complicate this rule. First of all, **the decline of an endemic species may remain unobserved** if the invasive species is very similar to the endemic one. The European blue mussel was able to invade northern California and caused declines in the population of the native *Mytilus trossulus* without being noticed, due to the morphological similarity of the species (Geller, 1999). *Spartina anglica* – an example of recent sympatric speciation, where hybridization led to a new successful invasive species (Ellstrand & Schierenbeck, 2000) – was able to invade territory that its parental species cannot colonize and rapidly displaced other native species (Huxel, 1999). Moreover, different cryptic lineages within an invasive species, for instance in the drosophilid species *Zaprionus indianus* (Yassin, et al., 2008), may also require **different strategies for control**, because important differences in life histories exist between the species.

Single species are often used as a **bio indicator of pollution, heavy metal contamination or environmental degradation**. The use of one species will be more accurate than relying on a complex of species (for instance cryptic species) (Geller, 1999). **Cryptic species may exhibit unique responses** to, for instance, heavy metals, suggesting differential tolerances at contaminated sites (as seen for marine copepods (Rocha-Olivares, et al., 2004)). It is thus extremely important to know exactly which species one is monitoring. The blue mussel (*Mytilus edulis*) is a commercially important species and is frequently used to monitor pollution. The morphospecies comprises three different cryptic species, with different life-history traits. Ignoring these differences may lead to inaccurate biomonitoring results, which

may result in human consumption of high levels of heavy metals or other pollutants (Geller, 1999; Bickford, et al., 2007).

## Nematodes: our model group and species

Increasing our understanding about differences in ecology of cryptic species and their coexistence is important and a wide range of organisms may be used to study this. **Roundworms** are a group within the Protostomia – Ecdysozoa. Their body length can range from 0.3 mm to over 8 meters and they can be parasitic or free-living. Estimates of global species diversity of free-living nematodes range between 500,000 to 1 million for terrestrial species (Briggs, 1994) and $10^5$ to $10^8$ for marine species (Coomans, 2002; Lambshead & Boucher, 2003; Appeltans, et al., 2012). True diversity may even be higher, as these estimations are largely ignoring cryptic diversity. Nematodes have a digestive canal which extends from the mouth to the anus and possess digestive, nervous, excretory, and reproductive systems (always sexual), but they lack a discrete circulatory or respiratory system (Malakhov, 1986). **Nematodes have successfully adapted to nearly every ecosystem** (Yeates & Bongers, 1999; Lambshead, 2004; Maslen & Convey, 2006; Van Gaever, et al., 2006).

Species identification in nematodes traditionally relies on morphological features, which lead to species characteristics often based on average measurements from one population of individuals (Powers, 2004). Very often, only a few morphological characters can be used for identification (Blouin, 2002). For this reason, **species boundaries in nematodes may be more easy to detect with DNA-based diagnostic methods** (Powers, 2004). Integrative taxonomists are now using combinations of molecular sequences, multivariate morphometric analyses and behavioural experiments (such as interbreeding tests) (Fonseca, et al., 2008). These combined methodologies, together with sampling a broader range of habitats, have led to a strong increase of newly described nematodes. In the last decades, more than half of the known species diversity in *Caenorhabditis* was discovered by collecting worldwide samples of rotten fruit (Kiontke, et al., 2011) and by an increased use of genetic techniques (Felix, et al., 2014). Cryptic species have been discovered in other terrestrial (e.g. Kanzaki, et al., 2012), parasitic (Obendorf, et al., 1991; Chilton, et al., 1995; Hoberg, et al., 1999; De Ley, et al., 2007) freshwater (Ristau, et al., 2013) and marine nematodes (Derycke, et al., 2005; 2007a; Bhadury, et al., 2008; Derycke, et al., 2008a; Fonseca, et al., 2008; Derycke, et al., 2010). The **marine** realm (next to tropical rainforests)

comprises some of the most species-rich habitats on Earth, which may **act as a breeding ground of cryptic speciation,** because many of the organisms are involved in specialized interspecific interactions (Bickford, et al., 2006). Moreover, marine species rely on chemical cues for these interspecific interactions and also for mate choice (Derycke, et al., 2013). Selection on these non-visual traits may lead to cryptic speciation (Willig, et al., 2003; Bickford, et al., 2007). Moreover, heterogeneity in time, topography, chemistry, oceanography, … renders the marine environment a very heterogeneous environment (Kaiser, et al., 2005).

In addition to the high global marine species diversity, **local diversity is generally also high** (usually several tens of species $m^{-2}$) for nematodes (Lambshead & Boucher, 2003). Nematode communities mostly contain many similar species belonging to the same functional groups (Bongers & Bongers, 1998). Finally, evidence for **co-occurring cryptic nematode species** in marine and terrestrial environments (Derycke, et al., 2005; 2007a; Derycke, et al., 2008b; Derycke, et al., 2010; Kiontke, et al., 2011) was found. Interactions between different nematode species are common and both facilitative – i.e., the presence of one species improves the occurrence of another – and inhibitory interactions exist (e.g. Ilieva-Makulec 2001; De Mesel, et al. 2006; dos Santos, et al. 2009). All these aspects render nematodes an ideal system to study mechanisms allowing coexistence of taxonomically and/or functionally related species, including cryptic species.

Many coexistence paradigms rely partly on dispersal (see above). But is dispersal common in marine nematodes? Small eukaryotic species (<1 or a few mm) are thought to lack geographical barriers because of their minute size and large populations that facilitate their dispersal (Šlapeta, et al., 2006). This dispersal is mostly passive, **with the water flow** following erosion from sediments or through **rafting on algae** (Thiel & Gutow, 2005). Nematodes may anchor themselves to the algal surface by secretions or caudal glands and even their eggs may be glued to the algal surface (Micoletzky, 1922). The dauer stage of nematodes may also be important in this stage, as dauer larvae are able to resist the harsh conditions during the dispersal event (Burnell, et al., 2005). Dispersal is not always a completely passive process: some nematodes are capable to **partly control their settlement** after dispersal (Ullberg & Olafsson, 2003; Schratzberger, et al., 2004; Guilini, et al., 2011; Lins, et al., 2013; Mevenkamp, et al., 2016). Active dispersal over large distances is considered non-existent because of their **small size, their poor swimming capacity and the lack of pelagic larvae** (Boeckner, et al., 2009). Nevertheless, nematodes may migrate

laterally through sediments (Schratzberger, et al., 2004; Ullberg, 2004; Gallucci, et al., 2008) and some evidence for active swimming has been found in nematode species from tidal flats (up to distances of >1m) (Thomas & Lana, 2011). Such passive and active dispersal, may be important to avoid unfavourable conditions, such as inter- and intraspecific competition and may enhance coexistence. Hence, studying **dispersal in nematodes** may be very interesting not only to explain some of the coexistence paradigms, but also to increase our knowledge of dispersal in meiofauna.

**The *Litoditis "marina"* species complex**

The model species complex of this PhD research is *Litoditis marina* (Sudhaus, 2011), formerly known as *Rhabditis marina* Bastian, 1865 or *Pellioditis marina* (Bastian, 1865) Andrassy, 1983. At least **ten cryptic species have been found within this morphospecies complex** (Derycke, et al., 2008b), for this reason further reffered to as *Litoditis "marina"* or *L. "marina"*. At the time of the discovery of this cryptic diversity in *L. marina*, the morphospecies was still named *Pellioditis marina*, hence the Pm abbreviations used to label each cryptic species. Four of them (Pm I, Pm II, Pm III and Pm IV) frequently occur along the south-western coast and estuaries of The Netherlands. These species show concordant **molecular divergences at nuclear and mitochondrial loci** (Derycke, et al., 2008a; Fonseca, et al., 2008). Pm I and Pm IV are the two most closely-related cryptic species (Derycke, et al., 2005)(Fig.3). The species lack single distinctive morphological characters (Fig. 4), but a multivariate morphometric analysis already showed that the cryptic species are not morphologically identical (Fonseca, et al., 2008). Moreover, breeding experiments between different species of the cryptic species complex did not result in any offspring (Fonseca, et al., 2008; Veltjen, 2012).

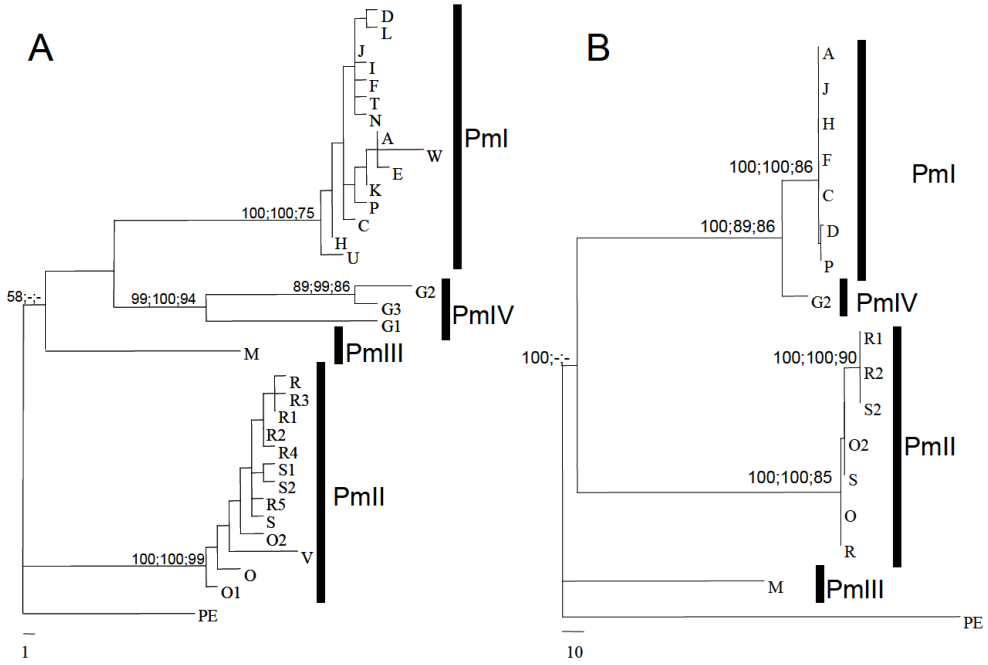



Figure 3: Phylogeny of *Litoditis "marina"* cryptic species. Maximum parsimony trees for (A) COI and (B) ribosomal ITS sequences. The 3 bootstrap values on branches represent (from left to right) maximum parsimony, neighbour-joining and maximum likelihood. Pm I, Pm II, Pm III and Pm IV: 4 clades; congener *Pellioditis ehrenbaumi* (PE) was used as outgroup species (senior synonym: *Buetschlinema nidrosiense*) (Derycke, 2005).


Figure 4: Drawings of the four cryptic species of *Litoditis "marina"* (Fonseca, et al., 2008): (a-c) Pm I, (d-e) Pm IV, (f-h) Pm III, (i-j) Pm II. Upper part of the body is illustrated in a, d, g and i. The tail of a male in c,e,h and j and the tail of a female in b and f. Scale bar: 60 µm..

*Litoditis "marina"* belongs to the same family (Rhabditidae) as the well-studied model organism *Caenorhabditis elegans* and is almost as **easy to handle and cultivate**. Its fast generation time (4 to 5 days) and high reproductive output make it a good model organism **to study several generations** in a short time. All species are gonochoristic and parthenogenesis has hitherto not been observed. The life cycle consists of four juvenile stadia and a metabolically less active but behaviourally 'normal' dauer larva may be formed when conditions turn unfavourable (Bongers, 1990).

*Litoditis "marina"* is a **bacterivorous nematode living on both living and decaying algae in the littoral zone** (Moens & Vincx, 2000a). They can reach huge abundances on piles of macroalgal wrack washed ashore on sheltered beaches (Somerfield & Moens, pers. observ.), but are also common and abundant on sheltered intertidal stands of living macroalgae in estuarine habitats. These typically comprise *Fucus* holdfasts on rocks scattered in muddy or fine sandy sediments or at the basis of piers or dykes (see fig. 5a). Such algal thalli are often covered with microbial biofilms and with *Enteromorpha* and have variable amounts of sediment embedded in the biofilms. Most of our observations stem from such living algal stands rather than from algal piles washed ashore, although an earlier study focused on the colonization-extinction dynamics of *Litoditis marina* on stranded wrack (Derycke, et al., 2007). The wrack is a transient habitat with a variable 'life span' and abiotic characteristics (including hydrodynamics). The living algal stands are a more stable habitat: they are present whole year round in relatively stable abundances, but the biofilms and the abiotic environment are undoubtedly also quite variable in (micro)space and time. *Litoditis* may play a role in ecosystem functioning in both these habitats, because it may affect bacterial populations which determine decomposition, but also microbial biofilm formation (Freckman, 1988; Hubas, et al., 2010). Bacterial communities on the algae are the most important food sources for *L. "marina"*, but differ temporally and between species of algae (Lachnit, et al., 2011). Estuarine intertidal environments are typically very **heterogeneous** environments, with **salinity and temperature**, among other factors, showing fluctuations at different temporal scales, from daily to seasonal (Moens & Vincx, 2000b; Kaiser, et al., 2005), but also spatially. Salinity and temperature can even vary between two nearby algae patches because of variation in the actual topography of the algal holdfast (for instance in shallow gullies and puddles, or at slightly different levels in the intertidal). They may even vary within a single algal patch or holdfast, because the macroalgae themselves also present a heterogeneous environment: the deterioration of decomposing algae creates **temporal**

**variability in resource availability, nutritional quality, and bacterial assemblage composition**, but also spatial variability in abiotic fluctuations, such as temperature, salinity and desiccation. The same holds for holdfasts of living algae, where thalli may cover and shelter other thalli and thus provide different conditions with different ranges of variation. Hence, the degree of daily variation in temperature, for instance, may differ between, but even within single algal patches. Moreover, at one location, high abundances of algae – mostly in dense patches- can be found (fig. 5a). The macroalgae create in this way a **spatially heterogeneous** environment, because nematodes can for instance hide in different levels of piles of algal wrack or even in different structural features such as receptacula and floating bladders. The very dynamic environment described above, makes it extremely difficult for a population to reach an equilibrium state, certainly on decomposing algae. On the stands of living algae, however, populations may be more stable. Nevertheless, the fluctuations in abiotic conditions and conditions of the microbial biofilm can also result in continuously changing populations.

It is common to find **two or three of the cryptic species** Pm I, Pm II, Pm III and Pm IV **co-occurring** on a **local** (one specimen of macroalgae; De Meester, et al., unpublished data) and **regional scale** (on different specimen from the same location, Derycke, et al., 2006, see fig.5b). Moreover, differences in species composition were found between different locations along a salinity gradient in the littoral zone of the south-western coast and estuaries of The Netherlands, indicating the possible influence of abiotic conditions on species composition (Derycke, et al., 2005). Species abundances also differed depending on the **seasons**, which may also indicate that abiotic conditions, such as temperature, influence the cryptic species. In the fast changing and disappearing (as a consequence of the deterioration of decomposing algae) habitat **colonization** to new algae patches is extremely important, and was already proven to happen faster when other algae patches were nearby (Derycke, et al., 2007b).“

A)

B)

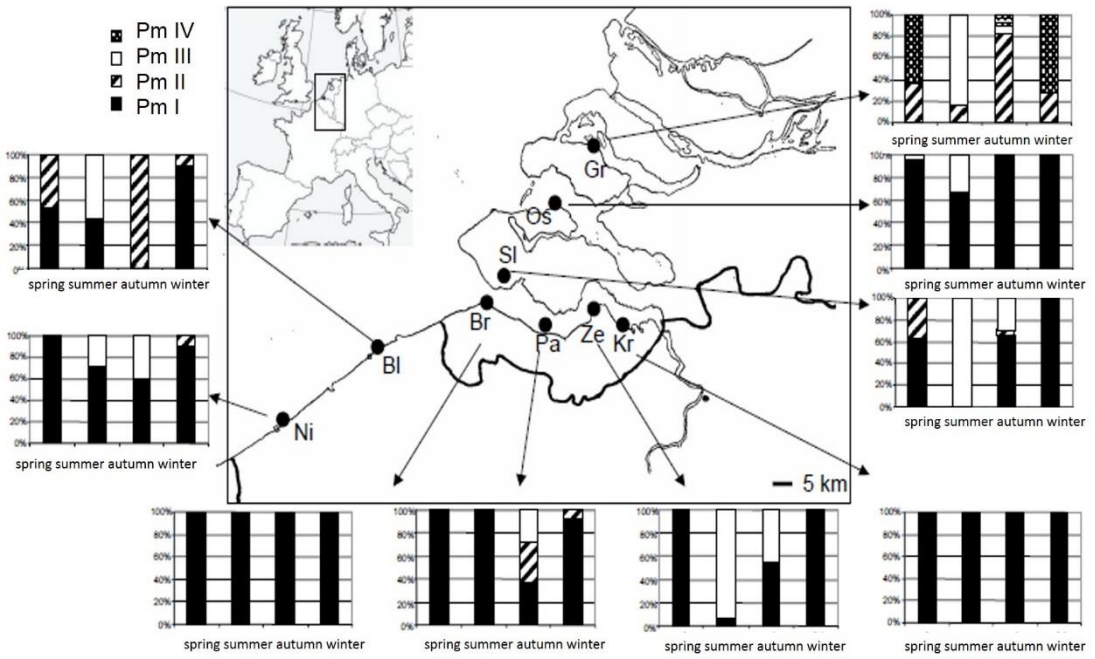



Figure 5: A) picture of a typical habitat of *L. "marina"* (Paulina mud flat, WesterScheldt, The Netherlands, February 2015): one specimen of macroalgae represents the local scale, different algal patches represent the regional scale. B) Distribution of 4 cryptic species of *Litoditis "marina"* (Pm I, Pm II, Pm III and Pm IV) along the Belgian coastline and the Scheldt estuary in The Netherlands. For each location, a stacked column graph indicates the percentage of each species occurring at a regional scale (different algae patches in one location) in spring 2003, summer 2003, autumn 2003 and winter 2004. Note the small proportion of Pm3 during winter 2004 in Br. Sample abbreviations: Ni = Nieuwpoort, Bl = Blankenberge, Br = Breskens, Pa = Paulina, Ze = Zeedorp, Kr = Kruispolderhaven, Sl = Sloehaven, Os = Oosterschelde, Gr = Grevelingen (from Derycke, et al., 2006).

## Outline of the thesis

In this study our main aim was to investigate the coexistence of four cryptic species of *Litoditis "marina"* (Pm I, Pm II, Pm III and Pm IV) and interspecific interactions between them. More detailed studies on ecological characteristics of the species may help to reveal some of the mechanisms behind their co-occurrence in natural environments.

In **chapter II** we hypothesised that species composition will not differ when interspecific interactions between the species were present and that salinity will not change these interactions. **Competition between the species** is investigated in a closed environment without dispersal possibilities. If competition between the species occurs, there will be no complete niche differentiation possible in our experimental set-up. Moreover, if **salinity changes competitive abilities**, there is a heterogeneous competitive environment for the organisms, and spatial storage or mass-effects paradigms may be important to achieve coexistence. The results of this chapter have been published as *"Salinity effects on the coexistence of cryptic species: A case study on marine nematodes" (De Meester, N., Derycke, S., Bonte, D. & Moens, T. (2011) Marine Biology 158: 2717-2726).*

Cryptic species of *L. "marina"* show sympatric distributiuons in the field, so **some mechanism(s) for their coexistence** have to exist. In the next three chapters we will focus more on the niche theory as a possible coexistence mechanism. Our hypothesis in **chapter III** is that the different cryptic species do not differ in their life-history traits -such as juvenile development time, fecundity and total population development- and their response on abiotic factors (salinity and temperature). In this way we tested if **niche differences** between the species are absent. If niche differences do exist but also some niche overlap is found, a kind of spatial storage paradigm may be possible. These results have been published as *"Temperature and salinity induce differential responses in life histories of cryptic nematode species" (De Meester, N., Derycke, S., Rigaux, A. & Moens, T. (2015) Journal of Experimental Marine Biology and Ecology 472: 54-62).*

In chapter III, constant abiotic conditions were tested, which does not reflect the natural conditions in which the nematodes live. For this reason, daily fluctuating temperatures were compared with constant temperatures in **chapter IV**. Moreover, environmental fluctuations may be important to achieve coexistence. We hypothesised that **temperature fluctuations** did not have an effect on **population performance** compared with constant temperature and that temperature fluctuations did not influence **interactions** between the cryptic species. The

results of this chapter were published as *"Daily temperature fluctuations alter interactions between closely related species of marine nematodes" (De Meester, N., dos Santos, G.A.P., Rigaux, A., Valdes, Y., Derycke, S. & Moens, T. (2015) PLoS One 10: e0131625).*

In **chapter V** we investigate niche differentiation in light of the **resource use of the different species.** Morphospecies that have been seen as dietary generalists are often complexes of cryptic species that may be dietary specialists (Bickford, et al., 2007). These differences may be subtle and not yet discovered (Ortells, et al., 2003) and may contribute to coexistence. **Next Generation Sequencing** on the microbial diversity within nematode organisms was conducted to investigate possible differences in food resource use of the species. We tested the hypothesis that the microbiome of cryptic species do not differ in microbial diversity. For this, we investigated differences in both the microbiome 'sensu stricto', containing the commensal bacteria in the gut and on the cuticle of the nematodes, and the microbiome 'sensu lato', which comprises the bacteria also related to food. The results of this chapter are in press as *"Coexisting cryptic species of the* Litoditis marina *complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability". (Derycke, S., De Meester, N., Rigaux, A., Creer, S., Bik, H., Thomas, W.K. & Moens, T. (2016) Molecular Ecology* : doi: 10.1111/mec.13597*)).*

While in the previous chapters, the experiments were always conducted in environments without dispersal oppurtunities, the next two chapters incorporate **active dispersal**. Dispersal is an important factor in all metacommunity paradigms (regional coexistence), as species may control when they are leaving a patch (for instance when interspecific interactions become too high). In **chapter VI**, we hypothesised that cryptic species do not show differences in their active dispersal abilities. The results of this chapter have been published as *"Differences in time until dispersal between cryptic species of a marine nematode species complex" (De Meester, N., Derycke, S. & Moens, T. (2012) PloS One, 7, e42674).*

In **chapter VII** we test whether **dispersal possibilities can result in coexistence of the cryptic species** and what the exact drivers of dispersal are for these species when they occur together. In an experimental set-up we combined all four cryptic species but gave them the chance to disperse away from the interspecific interactions. The tested hypotheses were that dispersal will not have an influence on the assemblage dynamics and that food availability and intraspecific and interspecific competition do not alter the moment of dispersal. These

results have been published as *"Active dispersal is differentially affected by inter- and intraspecific competition in closely related nematode species" (De Meester, N., Derycke, S., Rigaux, A. & Moens, T. (2015) Oikos 124: 561-570).*

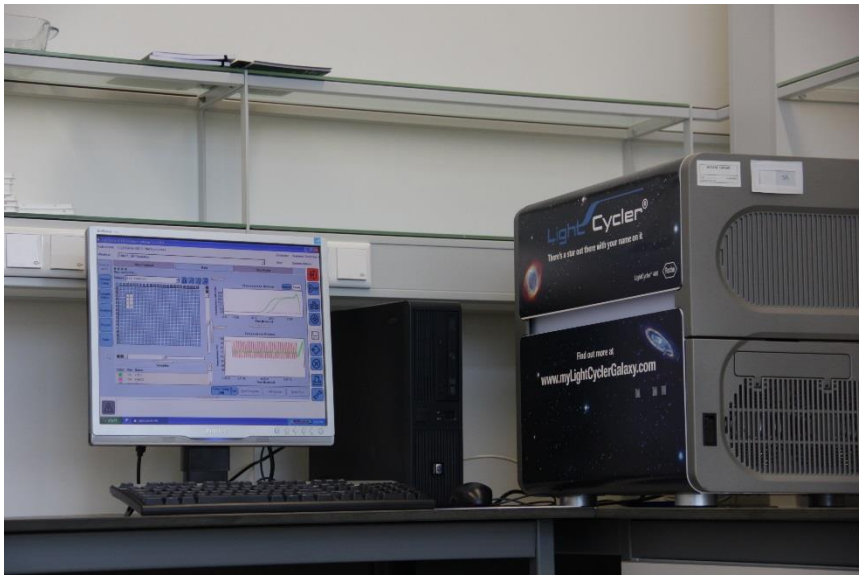
If there are niche differences between the cryptic species, these could potentially translate in functional differences, in which case we may expect that cryptic species could play different roles in **ecosystem functioning**. Because *L. "marina"* can substantially impact **decomposition processes** (see above), we hypothesised in the last regular part of this work (**chapter VIII**) that different cryptic species have no different functional roles in decomposition, and as a consequence in ecosystem functioning. We did so by studying the effect of the different cryptic species on the rate of loss of organic matter from decomposing algae and on the activity of two key extracellular enzymes involved in the degradation of this phytodetritus. These results are accepted as "*Cryptic diversity and ecosystem functioning: a complex tale of differential effects on decomposition" (De Meester, N., Gingold, R., Rigaux, A., Derycke, S. & Moens, T., Oecologia).*

Finally, in the last chapter we integrate and discuss the obtained results on niche differentiation, competition, dispersal differences and effect on decomposition (overview in Table 1) to try to build a conceptual/comprehensive framework on how coexistence may be achieved for cryptic species with sympatric distributions.

Table 1: Overview of the different topics and specifications of the different chapters of the PhD thesis.

| **Main topic** | **Specification** | **Chapter** |
|---|---|---|
| Competition | Interspecific interactions in closed microcosms and the effect of salinity on these interactions | Chapter II |
| Niche differentiation | Life-history traits at different salinities and temperatures | Chapter III |
| | Effect of fluctuating versus constant temperature on population performance and interspecific interactions | Chapter IV |
| | Resource use | Chapter V |
| Dispersal | Dispersal differences | Chapter VI |
| | Effect of dispersal on competition | Chapter VII |
| Ecosystem functioning | Effect on the decomposition process | Chapter VIII |

# CHAPTER II




# COMPETITION BETWEEN CRYPTIC SPECIES IN A HOMOGENEOUS ENVIRONMENT

---

Slightly modified from:

De Meester, N., Derycke, S., Bonte, D. & Moens, T. (2011) Salinity effects on the coexistence of cryptic species: A case study on marine nematodes. *Marine Biology,* 158, 2717-2726.

## Abstract

The coexistence of four cryptic species of *Litoditis "marina"* (Nematoda: Rhabditidae) at small geographical scale challenges ecological competition theory and was therefore studied in the laboratory at two different salinities, where their performance in combined cultures was compared to that in monospecies cultures. We found that three of the four cryptic species were able to coexist, but that interspecific interactions (competition and facilitation) were common. Salinity had an effect on these interactions, with a shift from contest to scramble competition. This shift may result from an increased population development of two of the four species at the lower salinity in the monospecific cultures. This experiment demonstrates that abiotic conditions may play an important role in achieving coexistence between cryptic species and can alter the interspecific interactions between them.

## Introduction

Behind the morphological similarity of many species hides considerable genetic diversity (e.g. Vrijenhoek, et al., 1994; Williams, et al., 2006; Fouquet, et al., 2007). This cryptic diversity implies that biodiversity in ecosystems is significantly higher than previously thought (Bickford, et al. 2007) and the consequences on ecosystem functioning are poorly understood. The coexistence of cryptic species at local scales (e.g. Trewick, 1998; Ortells, et al., 2003; Zhang, et al., 2004; Derycke, et al., 2006; Wellborn & Cothran, 2007) challenges traditional ecological competition theory, which implicitly expects competition to be most severe between closely related species (Darwin, 1859; Webb, et al., 2006; Violle, et al., 2011), leading to competitive exclusion under constant environmental conditions (Crombie, 1947; Webb, et al., 2002). Neutral dynamics, where species can persist together through non-equilibrium dynamics (Hubbell, 2005), and niche partitioning, where species coexistence is explained by differences in phenotype (Hutchinson & MacArthur, 1959; Hughes, et al., 2008), are two possible mechanisms which may explain coexistence of closely related species and together influence community structure (Leibold & McPeek, 2006). Whereas neutral dynamics – although it can potentially explain coexistence at short time scales– is usually considered important with respect to long-term coexistence (Chesson, 1991), niche partitioning remains the most plausible explanation for short-term coexistence. The absence of obvious phenotypic and/or ecological differences between cryptic species at first glance renders niche differentiation unlikely. Nevertheless, despite the fact that ecological and functional differences between cryptic species are largely unknown, closely related

sympatric species can display different environmental preferences (Knowlton, 1993), and hence ecological heterogeneity may facilitate the coexistence of the cryptic species (Leibold & McPeek, 2006). In environments with fluctuating dynamics, competitively inferior species may persist because they are temporarily favoured by specific conditions (Begon, et al., 1996). Under these circumstances, the results of interspecific interactions will depend on fluctuations in the abiotic (Crombie, 1947; Dunson & Travis, 1991) or biotic environment (e.g. food availability, predation, intraspecific competition (Jensen, et al., 2001)).

Cryptic speciation has recently been discovered in marine nematodes (e.g. Derycke, et al., 2005; 2007a; Bhadury, et al., 2008), the most abundant metazoan phylum in marine sediments (Coomans, 2000). The high diversity of species at both global (estimates ranging from $10^5$ to $10^8$ nematode species worldwide (Lambshead & Boucher, 2003)) and local (usually several tens of species $m^{-2}$) scales, their roles in decomposition processes (De Mesel, et al., 2006), and the different functional ecology of the species contribute to the importance of nematodes for ecosystem functioning (Coull, 1999). The coexistence of closely related species is important for the long-term stability of ecosystem functioning (Ettema, 1998), as ecologically similar species may compensate for each other when a species goes extinct (i.e. the redundancy hypothesis (Walker, 1992a)). Hence, coexistence of cryptic species may be important for ecosystem functioning. The marine nematode *Litoditis "marina"* (Sudhaus, 2011) – previous known as *Rhabditis (Pellioditis) marina* (Andrassy, 1983) (henceforth referred to as *L. "marina"*) – is a common bacterivore associated with decomposing macro-algae in the littoral zone of coastal and estuarine environments, a typically heterogeneous habitat, both temporally and spatially (Moens & Vincx, 2000a). Within the morphospecies *L. "marina"* at least 10 cryptic lineages can be found (Derycke, et al., 2008a). A detailed morphological study of four of these cryptic species (Pm I, Pm II, Pm III and Pm IV, not yet formally described) revealed significant morphological divergences which, however, only become apparent through a multivariate character analysis. Hence, there are no single distinguishing characters which could be used in a dichotomous identification key. These morphological divergences correspond well with the molecular divergences found at three independent loci (COI, ITS, D2D3) (Derycke, et al., 2008a; Fonseca, et al., 2008). No crossbreeding was observed between the two most closely related of these cryptic species (Pm I and Pm IV), hence it is unlikely that more distant species could hybridize (Fonseca, et al., 2008). Information about differences in ecology between these cryptic species, however, remains scarce. Preliminary studies indicate some differences in the food preferences of

these cryptic species, Pm I and Pm IV exhibiting very similar preferences for a number of bacterial strains, but differing in these preferences from Pm II and Pm III (Derycke, unpublished data). More detailed information on their feeding ecology, as well as information about their fitness under varying environmental conditions, including salinity, hitherto remains unknown.

These four cryptic species frequently occur along the south western coast and estuaries of The Netherlands, and sympatric occurrence of two or more of these species in point samples is rule rather than exception (Derycke, et al., 2006). Moreover, these four cryptic species display fluctuating abundances which may be linked to seasonal dynamics in the environment. These fluctuations may for instance be linked to differential tolerances for salinity and/or temperature conditions, two of the most conspicuous environmental variables in tidal environments. If these temporal dynamics can be explained by differential tolerances of the cryptic species to abiotic conditions, coexistence of these cryptic species would be feasible (Gómez, et al., 1995). Even if the cryptic species show considerable overlap in their environmental tolerances, abiotic variation can still be one of the factors shaping communities by influencing interspecific interactions between the species (Lowe, et al., 2006).

In this research, effects of salinity on the coexistence of four cryptic species of *L. "marina"* have been studied (Pm I, Pm II, Pm III and Pm IV). Salinity is one of the determinant factors in nematode diversity and community structure (Heip, et al., 1985), but its effects are mostly considered on a broader geographical scale. At a local scale, daily tidal fluctuations occur, with the highest salinity variations between low and high tide in the mid-estuary (Kaiser, et al., 2005). There are also seasonal variations, which in the North Sea may range between 19 and 36, with lower salinities during early spring and higher salinities during summer (Tietjen & Lee, 1972). Understanding effects of changes in salinity may also be important in view of climate change and its effect on community structure. Melting ice caps may result in water level rise and lower salinity (Dailidiene & Davuliene, 2006). Evidence for differential salinity optima in the cryptic species of *L. "marina"* can be derived from natural populations (Derycke, et al., 2006; 2008b), but is ambiguous. To study the effect of salinity on interspecific interactions, we need to investigate (a) differential salinity optima of the different cryptic species in absence of other species, (b) the effect of interspecific interactions on the dynamics of the different cryptic species, and (c) the interaction of the abiotic (salinity) and biotic (interspecific interactions) factors. If differential salinity optima

exist in the cryptic lineages of *L. "marina"*, we may expect that (a) cryptic species show different population abundances at different salinities, (b) these differences in abundances lead to differences in interspecific interactions, and (c) the outcome of these interactions is at least partly dependent on salinity.

## Materials & Methods

### Nematode cultures

Cultures of the different cryptic species were initially raised from single gravid females to ensure monospecificity, and maintained on sloppy agar media (Moens & Vincx, 1998) under standardised conditions (temperature of 20°C; salinity of 25) with unidentified bacteria from their habitat as food. Cholesterol (100 µL $L^{-1}$) was added as a source of sterols, because nematodes on a purely bacterial diet appear incapable of de novo synthesis of specific sterols (Vanfleteren, 1980). Nematodes for the experiments were harvested from cultures in exponential growth phase.

### Monospecific experiments

To study the effect of salinity on the population dynamics of the different cryptic species, monospecific cultures were reared in Petri dishes (5 cm inner diameter) with 4 mL of 1% bacto agar medium prepared with artificial seawater (Dietrich & Kalle, 1957) with a salinity of, respectively, 25 and 15. The pH of the medium was buffered at 7.5 – 8 with TRIS-HCl in a final concentration of 5mM. The addition of the buffer and the salt concentration of the agar increase the initial salinity by ca 1.2 units. Frozen-and-thawed *Escherichia coli* (strain K12) were used as food source and added every tenth day (50 µL of a suspension with a density of $3x10^{10}$ cells $ml^{-1}$).

The monospecific cultures were inoculated with five adult males and five adult females of a single cryptic species. Nematodes were manually picked up from the stock cultures, bathed in clean artificial seawater (salinity of 25) for one hour and placed randomly on the Petri dishes. Every treatment (i.e. lower and higher salinity) was replicated four times for every cryptic species. During the first eight days (representing at least one and at most two generations in all the treatments and species) the total numbers of adults, juveniles and eggs were counted daily. Afterwards, population dynamics were examined every fourth or fifth day until day 35 of the experiment, when a decrease in abundances of adult nematodes in all replicates – except one –occurred. This decrease probably resulted from a combination of

crowding and food depletion (dos Santos, et al., 2008). On the 15th and the 25th day the entire population was transferred to a larger Petri dish (resp. 8 cm i.d. and 15 cm i.d.) with new agar medium and proportional food availability.

**Combined experiments**

Interspecific interactions were tested by the use of combined cultures, in which all four cryptic species were simultaneously inoculated at equal numbers (five adult males and five adult females of each cryptic species). These combined cultures were prepared as described above, with the exception of food provision, agar medium and size of the microcosms: food (bacteria) was not added separately at a fixed concentration but allowed to grow on the agar medium. For this purpose, a mixture of bacto and nutrient agar in a 4:1 ratio was used. This medium ensures sufficient bacterial growth throughout (most of) the experimental test period, and also eliminates the need for addition of cholesterol (Moens & Vincx, 1998). Final concentration and other properties of the agar medium were the same as in the monospecific cultures and in the stock cultures of the four cryptic species. Food was sufficiently present and populations declined at the same time (around 35 days) in all treatments. This observation combined with the results of previous experiments (Moens, et al., 1996; Moens & Vincx, 2000b) in which monospecific populations showed similar adult abundances and generation times when cultivated on bacto/nutrient agar or on bacto agar with addition of a fixed amount of food, ensure that the differences in culture conditions had a negligible effect on the food availability and on nematode population growth.

Population/assemblage dynamics were studied every fifth day of the experiment by counting adults and juveniles. On every sampling occasion, ca. one third of the adults of each replicate was removed by handpicking and stored in acetone (70% - 95%) for later genetic identification. The repeated removal of adults was expected to have only a moderate impact on the population dynamics, as the removal was done randomly and only a few gravid females could already produce a substantial population due to the short generation time and the high reproductive success of the species.

Identifications of the cryptic species were initially performed by use of restriction enzyme analyses (Fonseca, et al., 2008) on the samples of 5, 10 and 25 days. Meanwhile, we developed a novel and faster qPCR-based method (Derycke, et al., 2012) and used this for the identification of nematodes from the samples of the 15th and the 35th day of the experiment. First a DNA extraction was performed, which was similar for both identification

methods. Individual nematodes were handpicked from the experimental cultures, transferred to sterile distilled water to remove traces of agar and then transferred to a 0.5 ml Eppendorf tube containing 20 µl lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % NP 40, 0.45 % Tween20). Tubes were frozen for 10 min at –20 °C, after which one µl of proteinase K (10 mg $ml^{-1}$) was added. Lysis took place in an Eppendorf Mastercycler gradient PCR machine at 65 °C for 1 h followed by 10 min at 95 °C. Finally, the DNA samples were centrifuged for 1 min at maximum speed (14000 rpm). Subsequently, 1 µl was used as template for qPCR. Identification of the four species with qPCR was done using the Lightcycler 480 System and the Lightcycler 480 SYBR Green I master kit (Roche Diagnostics). Species-specific primers (Table 1) were developed in the ribosomal internal transcribed spacer region (ITS). Following optimization of primer concentrations and cycling conditions, the qPCR mixture was prepared for a 20 µl reaction volume on 96 well plates using 10 µl LightCycler 480 SYBR Green I Master 2X solution, 3 µl PCR-grade water, 6 µl of each primer (final concentration of 1 µM for Pm I and Pm III, 500 nM for Pm II and 200 nM for Pm IV) and 1 µl of template DNA. The thermal cycling protocol comprised an initial denaturation for 10 min at 95 °C followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 20 s at 60 °C and extension for 20 s at 72 °C.

Table 1: Primer sequences for the four cryptic species (Pm I, Pm II, Pm III and Pm IV) of *L. "marina"* used in the qPCR protocol (Tm= melting temperature; GC= guanine-cytosine-content).

| Target | Primer sequence (5' → 3') | Amplicon lenght | Tm (°C) | GC (%) | Concentration (nM) |
|---|---|---|---|---|---|
| Pm I | F: CGCTGACCTTCACTGGAATTT | 135 | 53 | 45.45 | 1000 |
| | R: CCGACTCCGGTTCAACTCA | | 53 | 57.89 | |
| Pm II | F: GATCATCGCTGACCTTGG | 294 | 50 | 55.56 | 500 |
| | R: CGCACCATGTTGCCATGA | | 50 | 55.56 | |
| Pm III | F: AGCGGGGTGAAAGCCCA | 410 | 52 | 64.71 | 1000 |
| | R: CTGAACTAGAATGGGTACATTCA | | 52 | 39.13 | |
| Pm IV | F: CGATGGATGGTTTTCGCG | 134 | 50 | 55.56 | 500 |
| | R: GTGTATTGACGCTGTCCGTT | | 52 | 50.00 | |

**Data analyses**

Only data from 0, 5, 10, 15, 25 and 35 days of the monospecific cultures were retained for the statistical analysis in order to create a balanced design with the combined culture experiment. Furthermore, data of the monospecific cultures were randomly summed to create four fictitious assemblages for every treatment, where the four cryptic species are simultaneously present without affecting each other's population development. The total and individual nematode species' abundances at the start of the experiments were thus the same in the fictitious assemblages and in the combined cultures. This enables direct comparison of assemblage structure and abundance with the combined cultures and hence assessment of the interspecific interaction effects in our experiment. For this purpose, a Permutational Based Multivariate Analysis of Variance (PERMANOVA (Anderson, 2001)) on the basis of Euclidean distance with 999 permutations was used with the exact counts of adults of the assemblages (fictitious for the monospecific treatments vs. real for the combined treatment) as dependent variable and three fixed factors: time, salinity and presence/absence of interspecific interactions (i.e. monospecific vs. combined cultures). Significant terms and interactions were investigated using posterior pair wise comparisons within PERMANOVA. A SIMPER analysis was used to identify which species primarily accounted for the observed differences. PERMDISP (distance to the centroid) (Anderson, 2004) was executed to test the homogeneity of multivariate dispersions in order to discriminate between real location effects (output of PERMANOVA) and effects explained by differences in the multivariate dispersion for the significant factors. Moreover, this test was also used to check for the sphericity of the repeated measurement data.

One-way ANOVAs were executed in R (R Development Core Team, 2008) on the log-transformed time-averaged numbers of adults to compare abundances within one species or one treatment.

## Results

### Salinity and interspecific interaction effects on assemblage structure

Interspecific interactions, salinity and the interaction between them were important factors determining the structure of the assemblages (Table 2). This significant interaction was not caused by differences in multivariate dispersion since there is homogeneity of variances ($F_{3,93}$= 4.1952, P= 0.102). Pm III explained most of the variation (> 54 % in each group) in assemblage structure between the different groups (salinity x interspecific interactions), followed by Pm I for combined cultures (> 29 %) and Pm IV for monospecific cultures (> 15 %). Fig. 1 shows the effect of interspecific interactions and salinity on the time-averaged numbers of adults. In absence of interspecific interactions (monospecific cultures), Pm III and Pm IV had higher time-averaged abundances of adults at the lower salinity compared with the higher salinity (borderline significant results of one-way ANOVA: Pm III: $F_{1,7}$=5.97, P=0.050 (log-transformed data) and Pm IV: $F_{1,7}$=5.76, P=0.053). Interspecific interactions had no negative effect on the number of adults of Pm I at both salinities and of Pm III at the higher salinity. At the higher salinity it even had a positive effect on the numbers of adults of Pm I and Pm III (resp. increase of 110% and 119% in comparison with monospecific cultures). Pm II and Pm IV clearly suffered from the interspecific interactions with decreased average numbers of adults at the higher salinity (resp. decrease of 40% and 47% compared with monospecific cultures) and even more so at the lower salinity (resp. decrease of 82% and 86% compared with monospecific cultures). Fig. 2 illustrates that Pm IV was completely absent from the assemblages at both salinities after 35 days and that at a lower salinity a bottleneck occurred after ten days.

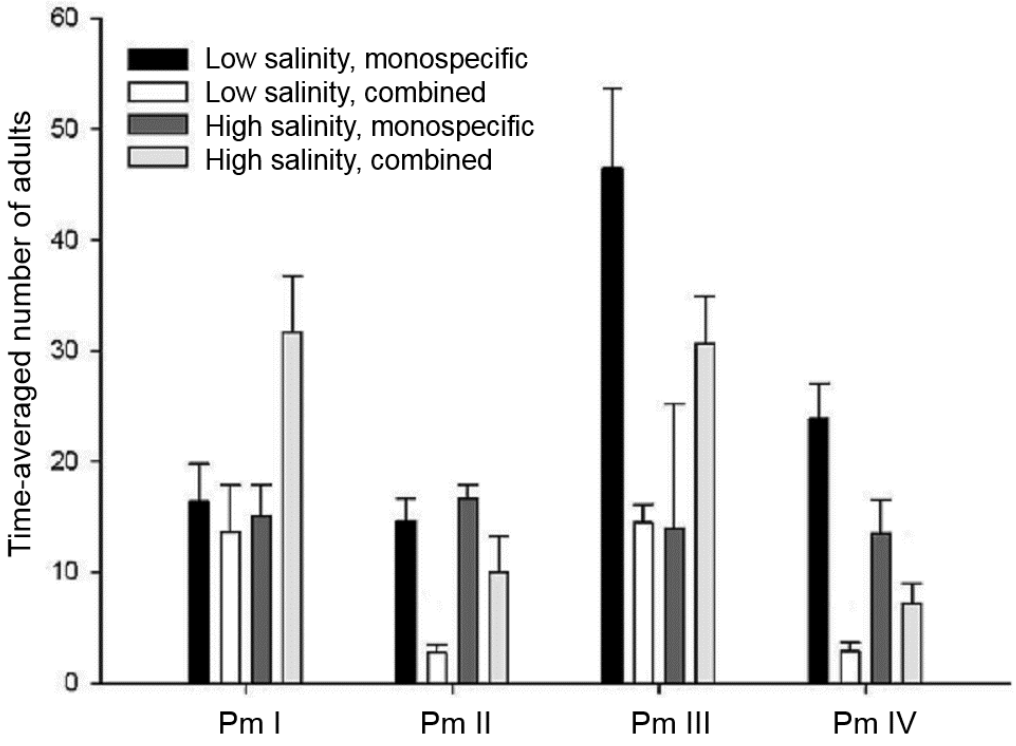



Figure 1: Time-averaged abundances of adults of the four cryptic species of *Litoditis "marina"* (mean ± SE) in monospecific and combined populations (four cryptic species together) at the two different salinities (low salinity: 15 vs. high salinity: 25) (n= 4 per treatment).

Table 2: PERMANOVA results from the analyses of counts of adults of the 4 cryptic species of *L. "marina"* as a function of time, interspecific interactions and salinity (* = P < 0.05).

| Source | df | SS | MS | F | P(perm) |
|---|---|---|---|---|---|
| **Time** | 5 | 33142 | 6628.4 | 1.7164 | **0.001** |
| **Interspec. interactions** | 1 | 9148.3 | 9184.3 | 6.535 | **0.001** |
| **Salinity** | 1 | 4048 | 4048 | 2.8803 | **0.048** |
| **Time*Interspec. interact.** | 5 | 30665 | 6133.1 | 4.3639 | **0.002** |
| **Time*Salinity** | 5 | 8821.9 | 1764.4 | 1.2554 | 0.231 |
| **Interspec. interact.*Salinity** | 1 | 17995 | 17995 | 12.804 | **0.001** |
| **Time*Interspec. interact.*Salinity** | 5 | 10834 | 2166.9 | 1.5418 | 0.125 |

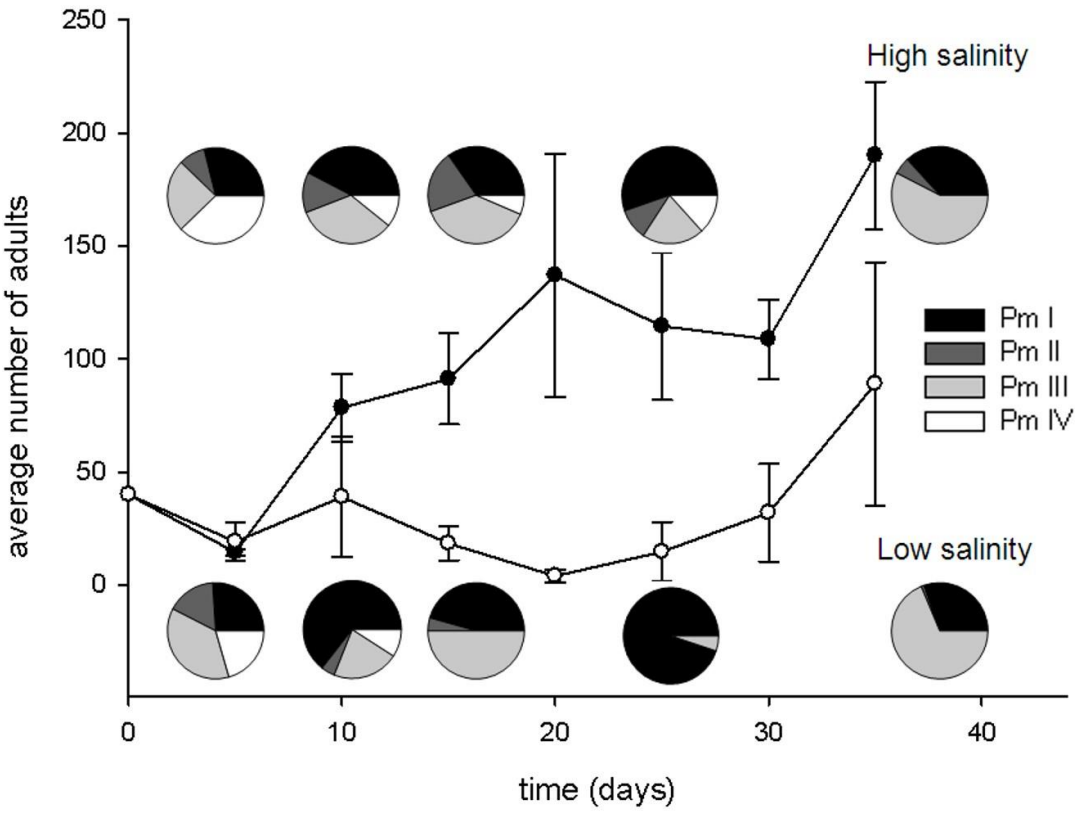



Figure 2: Assemblage dynamics for the combined cultures (lines: total number of adults of all cryptic species together) at high salinity (filled symbols) and low salinity (open symbols), together with the proportional abundances (pies) of the four cryptic species of *L. "marina"* after 5; 10; 15; 25 and 35 days. Note: at 35 days, Pm II was still present, but in very low abundances (n = 4 per treatment).

**Time effects on assemblage structure**

Assemblages changed significantly over time (see Table 2). PERMDISP displayed lack of homogeneity of variances ($F_{5,91}$= 13.217, P= 0.001), with higher variability as time progressed. An MDS plot revealed that differences in dispersion occurred between the different time moments and explained part of the temporal variation in assemblage structure, combined with an explicit time effect (Fig. 3).

Therefore interactions of time with other factors must be interpreted cautiously, to discriminate between dispersion and location effects. No interaction between time and salinity was found, indicating that within the time frame of the experiment no changes in the effect of salinity on assemblage structure occurred. On the other hand, an interaction between

time and interspecific interactions was found (Table 2) and pair wise tests for the homogeneity of variances also revealed that dispersion effects were present (Table 3). At the start of the experiment the differences in assemblage structure were mostly explained by the high variability in dispersion between the populations. Later on (between 25 and 35 days) time as location effect clearly explained the differences in assemblage structure in the combined treatments. Assemblage structure changed differently over time for combined and monospecific cultures (Fig. 4). Pm III and Pm IV explained most of the variation in time for cultures without interspecific interactions (resp. > 73% and > 14% for each time moment). In the combined populations, Pm III and Pm I explained most of the variation in time (resp. > 49% and > 42 %), due to the high fluctuations in abundances between different time moments.

Table 3**:** Results for the pair wise tests for PERMANOVA and homogeneity of variances between the different time moments in monospecific and combined treatments.

| **Groups** | **Monospecific** | | | | **Combined** | | | |
|---|---|---|---|---|---|---|---|---|
| **(time)** | PERMANOVA | | PERMDISP | | PERMANOVA | | PERMDISP | |
| | t | P | t | P | t | P | t | P |
| **0,5** | 2.17 | **0.001** | 5.90 | **$1*10^{-3}$** | 3.43 | **0.001** | 5.24 | **$1*10^{-3}$** |
| **5,10** | 2.17 | **0.024** | 3.34 | **$1*10^{-3}$** | 2.57 | **0.002** | 3.64 | **$5*10^{-3}$** |
| **10,15** | 0.66 | 0.588 | 0.76 | 0.474 | 0.73 | 0.692 | 0.38 | 0.781 |
| **15,25** | 1.70 | 0.067 | 1.80 | 0.133 | 1.20 | 0.245 | 1.18 | 0.386 |
| **25,35** | 1.68 | 0.054 | 1.70 | 0.156 | 2.30 | **0.021** | 1.21 | 0.242 |

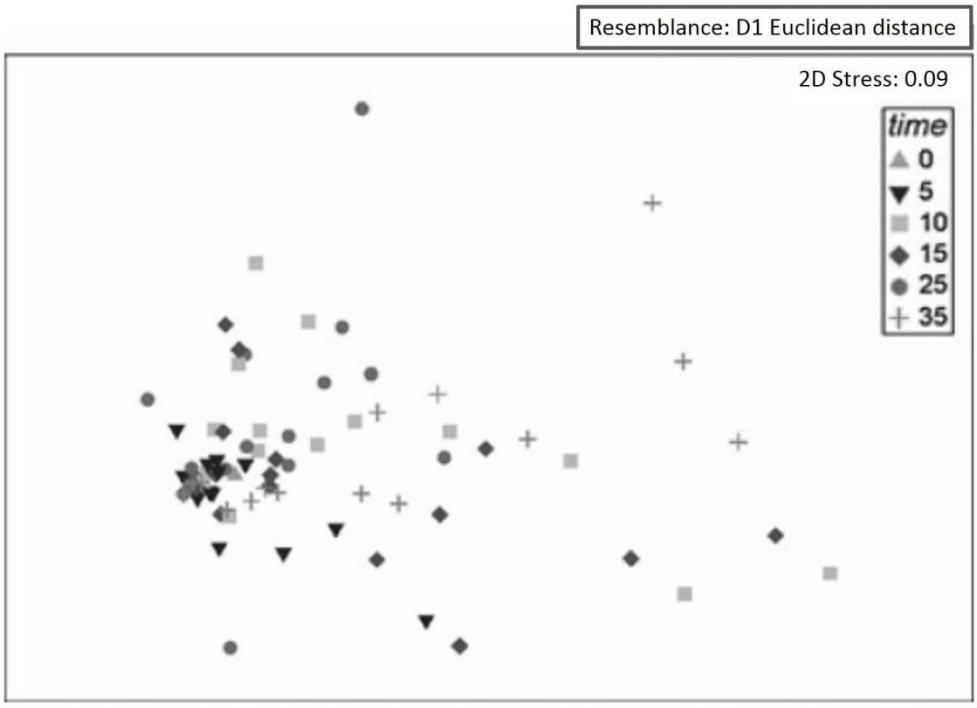



Figure 3**:** MDS plot showing the effect of time (in days) on the assemblage structure of the four cryptic species of *L. "marina"*. An explicit time effect combined with a dispersion effect can be found with higher variability between the different assemblages at later times (n = 96).

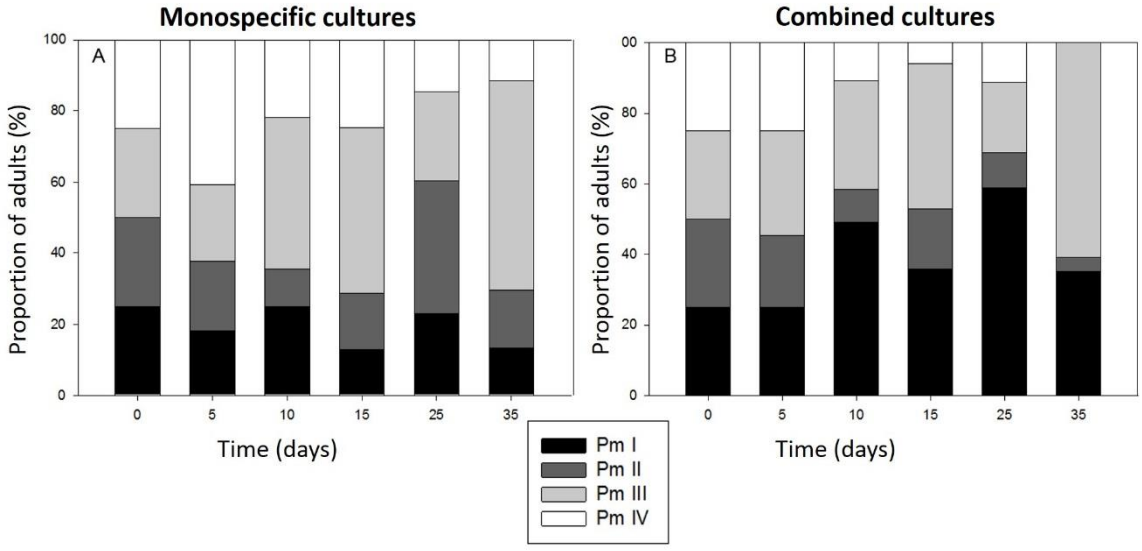



Figure 4**:** Average proportional adult abundances of the four cryptic species of *L. "marina"* in a) monospecific cultures and b) combined cultures as a function of time (n = 4).

## Discussion

In this experiment, coexistence between three of the four cryptic species of *L. "marina"* (Pm I, Pm II and Pm III) was found. The effect of salinity on this coexistence was studied, as salinity has proven to play an important role in structuring populations and communities of different marine and estuarine species (e.g. Capstick, 1959; Heip, et al., 1985; Williams, 1998; Westerbom, et al., 2002; Ortells, et al., 2003; Lowe, et al., 2006) and has an important impact on the life cycle of many species (e.g. Tietjen & Lee, 1972; Diaz & Bevilacqua, 1986; Anger, 1991; Højgaard, 1998; Moens & Vincx, 2000b). In the case of *L. "marina"* all cryptic species were able to cope well with the two different salinities in our monospecific experiments. Despite this overlap in salinity optima, salinity can still play an important role in achieving coexistence in two different ways: (a) if species have different salinity optima that only partially overlap, coexistence can be achieved in the zones of overlap, or (b) if the species have broad salinity optima, with only minor differences, even these minor differences can lead to changes in interspecific interactions between the species and thus result in coexistence (Lowe, et al., 2006).

**Different salinity optima did not easily predict the outcome of competition**

In the monospecific cultures some differences in relative population performance between the cryptic species at the two different salinities were found. Two of the four cryptic species (Pm III and Pm IV) showed higher population abundance at the lower salinity than at higher salinity (Fig. 1; and also confirmed in chapter IV). These differences in salinity optima between the cryptic species can help to achieve coexistence. We would expect that Pm III and Pm IV would dominate the combined cultures at the lower salinity. Fig. 4 shows that this is not completely true and contrary to the expectations: Pm IV showed very low average abundances and was completely excluded after 25 days. This shows that besides the differences in salinity optima other factors play a role in shaping the coexistence between the cryptic species. For Pm IV, higher population abundances in monospecific cultures only occurred at the beginning of the experiment and were followed by a sudden decrease after ten days (Fig. 5). This acceleration of development (first generation matured 1 day earlier than in the other species) may be the result of a short-term response to a sudden change in environmental conditions (Grainger, 1958), and can be important to achieve coexistence with the other cryptic species. If Pm IV is capable of reaching high abundances before other species become dominant and environmental fluctuations (fluctuations in salinity, temperature, … due to for instance the tides, weather conditions or seasons) occur on a

regular base, Pm IV can coexist with the other species. The higher abundance of Pm III at the lower salinity in monospecific cultures was evident over the whole time frame of the experiment and is in line with the high abundance of Pm III in the combined cultures.

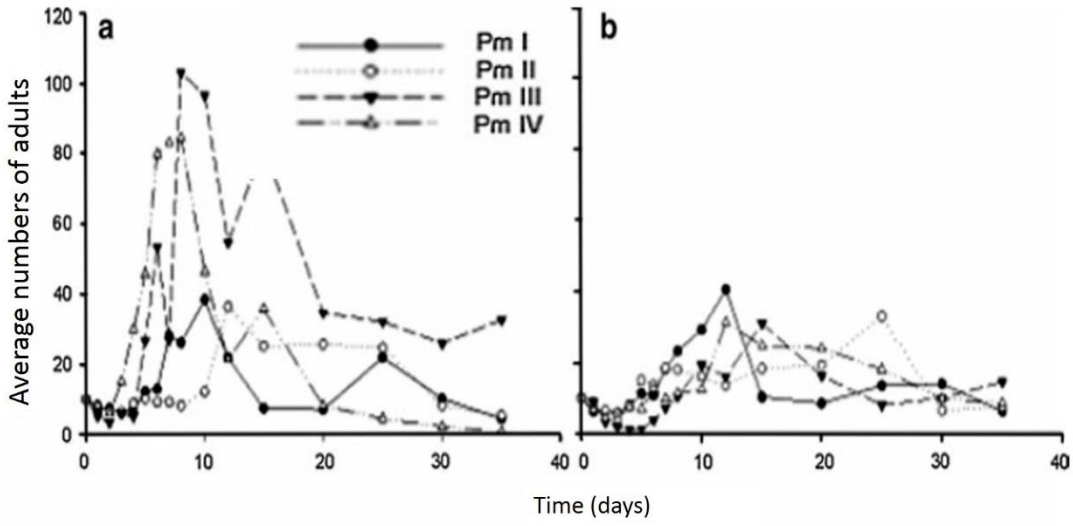



Figure 5**:** Average adult abundances of the four cryptic species of *L. "marina"* in monospecific cultures for the different salinities over time: a) low salinity and b) high salinity (n = 4)

Another possible explanation is that different optima between the cryptic species can influence the interactions between species and that coexistence is achieved in this way (Gómez, et al., 1997; Lowe, et al., 2006). The differences in population development in monospecific treatments at low salinity indeed suggest that the four cryptic species have differential salinity optima. To further assess this hypothesis, we first have to reveal the interspecific interactions between the cryptic species.

**Both negative and positive interactions occur between the cryptic species**

The most common interaction between species is competition. However, facilitative – i.e., the presence of one species improves the occurrence of another (Egler, 1954) – and inhibitory interactions may be equally important (e.g. Ilieva-Makulec, 2001; Cardinale, et al., 2002; Jonsson & Malmqvist, 2003; De Mesel, et al., 2006; dos Santos, et al., 2009). In our experiment interactions between organisms appeared between five and ten days in both salinity treatments; hence the first generation was not affected by the presence of other species. Later on, the presence of other species had a major effect on the survival of Pm II and Pm IV, with complete exclusion of Pm IV from all populations at both salinities. In natural populations in the area from which both species were isolated, Pm I and Pm IV have

hitherto never been found in sympatry. Pm I and Pm IV are phylogenetically the two most closely related species in the *L. "marina"* cryptic species complex (Derycke, et al., 2005), and preliminary experiments show that Pm I and Pm IV have very similar food preferences (Derycke, unpublished data), so the absence of coexistence between Pm I and Pm IV agrees with traditional ecological competition theory. Chemical repulsion by glandular secretions produced by the nematodes is also a possible mechanism that could explain the extinction of Pm IV in the combined cultures. Although chemical repulsion was already shown between marine copepods (Chandler & Fleeger, 1987), and nematodes are known to be sensitive to chemical cues (Huettel, 1986), almost no information on allelochemicals in free-living nematodes is present, making it impossible to confirm this hypothesis. In contrast to Pm IV, Pm II was still present at the end of the experiment in the combined cultures at both salinities, although in very low abundances. Pm II thus clearly suffered from the interaction with the other species, but the interactions were not strong enough to completely exclude Pm II from the assemblages.

In both salinity treatments Pm I and Pm III were highly abundant and positive interactions occurred at the higher salinity. The presence of other species possibly causes habitat amelioration – an important process in intertidal communities (Bertness & Leonard, 1997) – for instance as a result of the higher densities of nematodes influencing bacterial growth. In combination with the increased secretion of mucus by nematodes, which may transport bacteria to different spots, this may make food more available (Moens, et al., 2005).

**Effect of salinity: from contest to scramble competition**

But did salinity alter the outcome of these interactions? Interactions between an abiotic factor (i.e. salinity) and a biotic factor (i.e. interspecific interactions) have already been demonstrated in laboratory experiments on crustaceans (Foran, 1986; Bengtsson, 1987; Barata, et al., 1996) and were also present in this experiment. At lower salinity the interspecific interactions between the species became stronger, leading to a population crash in all four replicates (Fig. 2); only two of the four replicates recovered from this bottleneck. The assemblage structure of these two replicates at the end of the experiment was very similar to the assemblage structure at higher salinity (Fig. 6). These results indicate that salinity had no effect on the species composition of the assemblages, but rather had an effect on the type and strength of interspecific interactions. At the higher salinity two species became dominant while the other two suffered from the interactions. We can expect that at this salinity, resources (food, space, etc.) were unequally partitioned between the cryptic

species and that some species survived (here: Pm I and Pm III) at the expense of the others (here: Pm II and Pm IV). This is an example of contest competition. In contrast, at the lower salinity, all species initially suffered from the interactions, followed by either complete extinction of all species or survival of some of the species. This suggests that (most of) the species competed equally for the resources and none was initially able to fully meet its needs. If by chance some species die off earlier than the others, the remaining species may be able to recover. This is known as scramble competition (Nicholson, 1954; Lomnicki, 1988; Coulson & Godfray, 2007). Hence, our results strongly suggest that a shift occurred from contest competition at higher salinity to scramble competition at lower salinity. The initially higher population abundance of Pm IV at lower salinity (as seen in the monospecific cultures) may have increased interspecific interactions and may thus have contributed to this shift in the type of competition.

In this experiment, salinity did not only affect the population dynamics of the cryptic species, but also the interspecific interactions between them and the interaction between salinity and the interspecific interactions. Caution is due when linking these results to results from natural populations as in estuarine habitats, which are characterized by large abiotic fluctuations, adaptations to both longer-term, larger-scale and short-term, local-scale (tidal) salinity fluctuations are important. These adaptations to short-term salinity fluctuations were not studied in the present experiment, which renders comparison of our results with the species composition of natural populations difficult. Over all seasons, in the area from which all species for this study were isolated, Pm I, Pm II and Pm III co-occurred at locations with an average salinity around 25 and only Pm I and Pm III co-occurred at locations with an average salinity around 15 (Derycke, et al., 2005; 2006). These results are very comparable with the data from our experiment, but besides differences in salinity optima, also differences in optima to daily fluctuations or differences in optima to other environmental variables (temperature, food preference, etc.) between the species likely play an important role in the structuring of natural assemblages.

Differences in salinity did lead to differences in the population dynamics of some species (Pm III and Pm IV) and to differences in the interspecific interactions, suggesting that larger-scale; long-term salinity fluctuations may influence natural populations and communities. Furthermore, we can expect that seasonal dynamics in salinity can alter the species composition of the natural assemblages since shifts in cryptic species composition of *L. "marina"* occurred through the different seasons (Derycke, et al., 2006). To prove this

hypothesis, additional data from the field are necessary, but this experiment already shows that differences in salinity can have different results on the outcome of interspecific interactions, and that solely focusing on the effect of salinity on monospecific cultures is highly unsatisfactory.

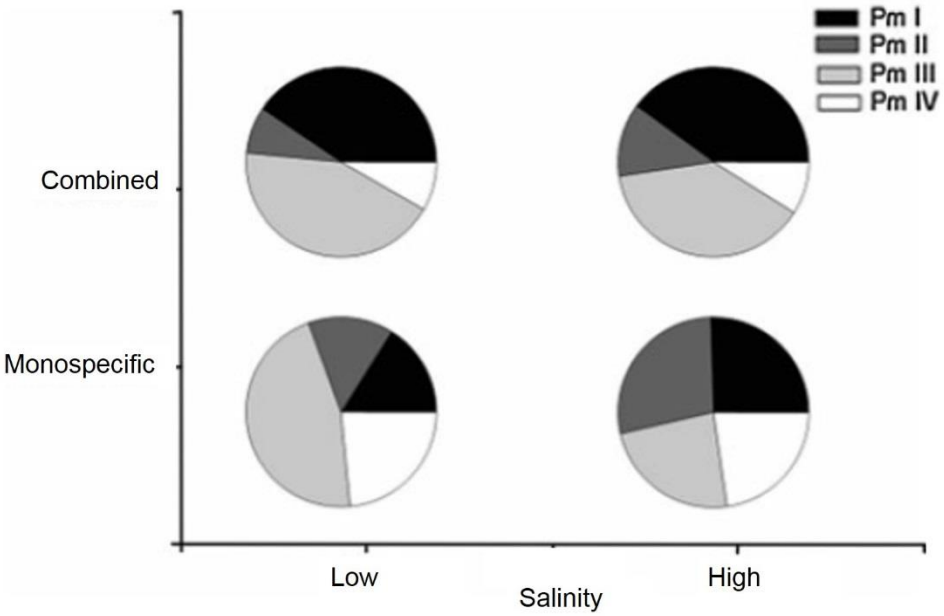
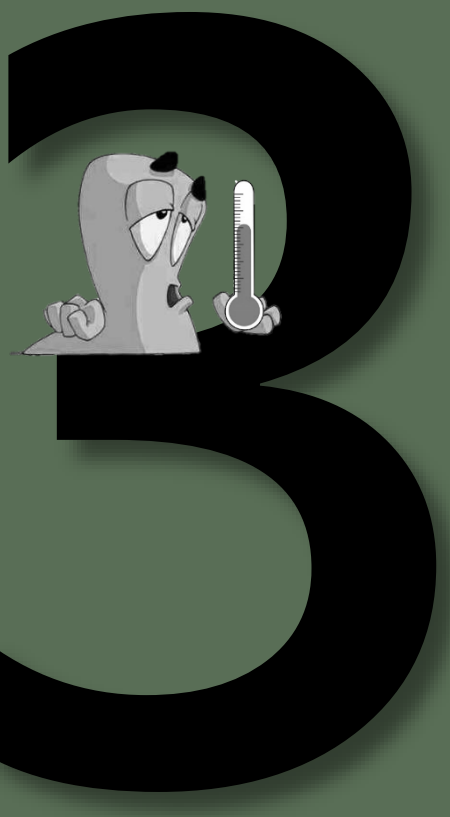



Figure 6: Time-averaged assemblage structure of the four cryptic species of *L. "marina"* for the different treatments (interspecific interactions x salinity, n = 4 per treatment).

## Acknowledgements

Funding for this research was obtained from the Flemish Fund for Scientific Research (FWO) through project 3G040407, and from Ghent University through projects 011060002 and 01GA1911W. S.D. is a postdoctoral fellow with the FWO. Annelien Rigaux is acknowledged for help with the monospecific experiments.



# CHAPTER III


# DIFFERENTIAL RESPONSES TO THE ABIOTIC ENVIRONMENT AMONG CRYPTIC SPECIES

---

Slightly modified from:

De Meester, N., Derycke, S., Rigaux, A. & Moens, T. (2015) Temperature and salinity induce differential responses in life histories of cryptic nematode species. *Journal of Experimental Marine Biology and Ecology*, 472, 54-62.

## Abstract

In the marine environment, many cryptic – morphologically similar but genetically distinct – species show sympatric distributions, which challenges traditional ecological competition theory. In the morphospecies complex of the bacterivorous nematode *Litoditis "marina"*, four cryptic species (Pm I, Pm II, Pm III and Pm IV) frequently occur with mostly two or more of these species together. This co-occurrence displays fluctuating abundances that have been linked to seasonal dynamics in the environment. In intertidal marine environments, salinity and temperature play an important role in species distributions. In the here presented experiments, the impact of these two abiotic variables on juvenile development time, fecundity and population development was investigated. Monospecific cultures were reared at three different temperatures: 15°C, 20°C and 25°C (salinity 25) and at two different salinities: 15 and 25 (temperature: 20°C) in 2 separate experiments. Our results showed that differences in life history are present between the four species: Pm III had a higher instantaneous fecundity than the other three species. Furthermore, differences in reproduction strategy were observed between the cryptic species: Pm II and Pm III were always oviparous in this experiment, while Pm I and Pm IV were mostly viviparous species. Abiotic factors affected the life history characters and the reproductive strategy of some of these cryptic species, with temperature clearly having a stronger effect than salinity. Temperature had an effect on juvenile development time for all species and a species-specific influence on population development. Pm III performed better at higher temperature, Pm II and Pm IV at lower temperature, whereas Pm I proved to be a more eurytherm species, which nicely correlates with their respective seasonal field distribution pattern in the south-eastern coast of the North Sea and adjacent estuaries. Juvenile development time was also influenced by salinity, with a shorter development time at the lower salinity for all cryptic species. Moreover, Pm III and Pm IV performed better at lower than at the higher salinity. Pm IV also changed its reproductive strategy, depending on the salinity. The effect of salinity was less clearly reflected in the geographical distribution of the four species. Different temperature preferences of the cryptic species on the other hand may result in niche differentiation and can lead to coexistence of these closely related species.

## Introduction

Biodiversity in many ecosystems appears significantly higher than previously thought due to the prominence of morphologically cryptic but genetically distinct species. This cryptic diversity is a wide-spread phenomenon within a broad range of taxa (Bickford, et al., 2007; Pfenninger & Schwenk, 2007). Many cryptic species show sympatric distributions in the marine environment (Knowlton, 1993). This coexistence challenges traditional ecological competition theory, which states that competition will be most severe between closely related species because of their ecological equivalence (Darwin, 1859; Webb, et al., 2002; Violle, et al., 2011). However, coexistence can be achieved through non-equilibrium dynamics (neutral dynamics: Hubbell, 2005), or when species show differences in phenotype and ecology (niche partitioning: Hutchinson & MacArthur, 1959; Hughes, et al., 2008). In the latter case, ecological heterogeneity could facilitate the coexistence of closely related sympatric species (Knowlton, 1993; Leibold & McPeek, 2006), and competitively weak species could persist because of spatially or temporally favoured specific conditions (Begon, 1996). However, knowledge about the ecology of cryptic species remains very scant despite the exponential increase in the documentation of cryptic diversity (Bickford, et al., 2007). Hypotheses about ecological niche differentiation between cryptic species are usually based on often limited information about geographical distributions (Ortells, et al., 2003; Rissler & Apodaca, 2007). Abiotic niche differentiation has already been invoked as a likely explanation for the coexistence of cryptic species in e.g. rotifers, sea urchins and nematodes (Palumbi & Metz, 1991; Ortells, et al., 2003; Montero-Pau, et al., 2011; Van Campenhout, et al., 2014).

Cryptic diversity has been observed several times in coastal nematodes (Derycke, et al., 2013). In marine sediments, nematodes are the most species-rich and abundant (densities between $10^5$ – $10^8$ individuals $m^{-2}$) metazoans (Heip, et al., 1985; Coomans, 2000; Lambshead & Boucher, 2003). Nematodes can play significant roles in microbial biofilm formation and in decomposition and nutrient recycling processes (Freckman, 1988; Hubas, et al., 2010). In the morphospecies complex of the bacterivorous nematode *Litoditis "marina"* (Sudhaus, 2011), formerly known as *Rhabditis marina* or *Pellioditis marina*, at least ten species have been found. Four of these cryptic *L. "marina"* species (Pm I, Pm II, Pm III and Pm IV; the 'PM' abbreviation originates from the old species name, but is kept to make comparison between different papers easier) frequently occur in the littoral zone of the south-western coast and estuaries of The Netherlands (Derycke, et al., 2006; 2008b).

These cryptic species lack distinctive morphological differences, but show molecular divergences at both nuclear and mitochondrial loci (COI, ITS, D2D3) (Derycke, et al., 2008b; Fonseca, et al., 2008), and crossbreeding between them has not been detected thus far (Fonseca, et al., 2008, Derycke, unpublished). Sympatric occurrence of two or more of these species on decomposing algae is rule rather than exception (Derycke, et al., 2005; 2008b). In addition to the temporal deterioration of decomposing algae, macroalgae also form a spatially heterogeneous habitat because they contain different structural features such as receptacula and floating bladders. Throughout the year, the four cryptic species display fluctuating abundances which may be linked to seasonal dynamics in the environment (Derycke, et al., 2006), possibly as a result of different environmental tolerances. However, the effect abiotic factors have on population abundances and life histories in these four cryptic species remains unknown. Salinity and temperature – two of the most conspicuous environmental variables in a tidal environment – show both daily and seasonal fluctuations. Salinity variations are highest between low and high tide in the mid-estuary and tidal fluctuations may be as high as 8 to 21 (Moens & Vincx, 2000b; Kaiser, et al., 2005). In addition, significant seasonal and small-scale local fluctuations occur; in the area of the present study these usually result in lower salinities during early spring and higher salinities during summer, and in even more prominent salinity fluctuations – both tidal and seasonal – in shallow gullies and puddles in the high intertidal (Moens & Vincx, 2000b). Salinity has been shown to have a relatively minor effect on juvenile development time, while more extreme salinity values can have a strong impact on juvenile survival of *Litoditis "marina"* (cryptic species unknown, Moens & Vincx, 2000b). Daily fluctuations in temperature are also present, but seasonal variation may be more pronounced. In the area of this study, maximum daytime temperatures can range from below 0°C to above 25°C. Increasing temperature has been shown to increase the fecundity and decrease the development time of marine and brackish-water nematodes (reviewed in Heip, et al., 1985; Vranken, et al., 1988). Moreover, the development time of *L. "marina"* was strongly affected by temperature (Moens & Vincx, 2000b). At the time of the latter study, the presence of cryptic species within *L. "marina"* was not yet known, and the molecular identity of the species used in that study is unknown.

In this paper, we combined results of a previous experiment dealing with salinity effects on competition between the cryptic species (De Meester, et al., 2011; chapter II) – in which we focused on the effect of salinity on competition between the species and did not yet describe

any life history traits – with those of a new and independent experiment investigating temperature effects on life history traits of the cryptic species. We discuss the impact of each abiotic variable on juvenile development time, fecundity and population development of the four cryptic *L. "marina"* species. The first two parameters will give more information about short-term responses, for instance a stress reaction to the differences in temperature or salinity between the stock culture and experimental microcosm conditions. The latter parameter integrates information over several generations and may therefore better reflect longer-term responses, in which species-specific differences in abiotic preferences and the effect of intraspecific competition (increasing densities) may become clear. The effects of temperature and salinity were studied in single stressor experiments. Although it is extremely important to know the effect of the combination of both abiotic variables (Breitburg, et al., 1998), detailed information about the effect of one abiotic variable on life-history traits can already give us insights in some of the complex processes in nature. A seasonal survey along the south-eastern coast of the North Sea and adjacent estuaries illustrated that Pm III was most abundant in summer, during which Pm II and Pm IV were mostly absent (Derycke, et al., 2006). Pm I was abundant throughout the year. Pm IV had a more restricted geographical distribution and was only found in a marine lake where fluctuations in salinity are less pronounced (Derycke, et al., 2006). Based on these observations, we expected to find differential effects of temperature on the life histories of the four species, with Pm I showing a minor effect of temperature on its life history characteristics. Pm II and Pm IV were expected to perform best at lower temperatures and Pm III at higher temperatures. Given the low salinity fluctuations in the lake where Pm IV occurs, it would be interesting to assess whether Pm IV is more sensitive to changes in salinity than the other species. Knowledge on the effect of abiotic conditions on the life history characteristics of the cryptic species may help to explain their distribution in natural environments and their coexistence.

## Material & Methods

### Nematode stock cultures

Stock cultures of the different cryptic species were raised from single gravid females and maintained on sloppy agar media (0.8 % 1:4 ratio of nutrient:bacto agar) prepared with artificial seawater with a salinity of 25 under standardised conditions (temperature of 20°C), with unidentified bacteria from their habitat as food (Moens & Vincx, 1998). Nematodes for the experiments were harvested from cultures in exponential growth phase.

### Temperature experiments

To study the effect of temperature on life history traits of the different cryptic species, monospecific cultures were reared in February – March 2013 in Petri dishes (5 cm inner diameter) with 4 mL of 1% bacto agar medium with a salinity of 25. The pH of the medium was buffered at 7.5 – 8 with TRIS-HCl in a final concentration of 5mM. The addition of the buffer increases the initial salinity by ca 1.2 units. Cholesterol (100 µL $L^{-1}$) was added as a source of sterols (Vanfleteren, 1980). Nematode cultures were incubated in the dark at three different (constant) temperatures: 15°C, 20°C and 25°C. While we acknowledge that this limited range of temperatures is insufficient for a complete picture of the effects of in situ seasonal temperature variation on *L. "marina"*, it may nevertheless help to explain differential effects of seasonal fluctuations within this cryptic species complex; 15°C represents mean temperatures for early autumn and late spring and 20°C for summer, whereas 25°C represents fairly common daytime temperature maxima during (mostly) summer. Each treatment was replicated four times for every cryptic species. Frozen-and-thawed *Escherichia coli* (strain K12) were used as a food source (50 µL of a suspension with a density of $3x10^9$ cells $ml^{-1}$). This dilution was obtained through dilution in ASW from a stock density of $3\times10^{11}$ cells $ml^{-1}$ (dos Santos, et al., 2008). This strain of *E. coli* has been commonly used as a single and adequate food source in different experiments with *L. "marina"* (dos Santos, et al., 2009; De Meester, et al., 2011 (chapter II); 2015b (chapter VII)). Moreover, dispersal experiments showed that all 4 cryptic species are able to detect *E. coli* (as food source) (De Meester, et al., 2012; chapter VI). On the 13$^{th}$ day of the experiment, the same amount of food was added to replenish the cultures. The plates for the temperature experiment were inoculated with four adult females and three adult males of a single cryptic species in each replicate microcosm. Only healthy, vivid animals were chosen for the experiment. Females were randomly chosen from the stock cultures and we took care to select young adult females which were recognisable by a combination of size, visible

presence of a vulva, and presence of at most a few eggs in the uterus. As such, we are sure to have selected females in an early phase or at the start of their reproductive period. Nematodes were manually picked up from the stock cultures, bathed in clean artificial seawater (salinity of 25) for one hour, and placed randomly on the Petri dishes. The total numbers of eggs and juveniles were counted after 24h; total numbers of nematodes were counted daily until the cultures reached the exponential growth phase, after which counting was done every 48 hours until day 21. When the first juveniles occurred (mostly after 24 hours), all adults were removed from the Petri dishes, which made it possible to determine the juvenile development time of the F1 generation.

**Salinity experiments**

This experiment was part of a previous experiment, in which monospecific cultures were raised at two different salinities (15 and 25). This limited range of salinities can still give us information about the geographical distributions of the cryptic species in the estuarine and lake habitats covered in earlier field inventories, since 15 is close to the lower end of the salinity range in which *L. "marina"* occurred, whilst 25 corresponds to a salinity at which all four cryptic species were commonly found (Derycke, et al., 2005; 2006). Agar medium and set-up of the experiment were the same as in the temperature experiments, except for the number of adults that were inoculated (five males and five females instead of three and four, respectively). Numbers of juveniles and eggs were counted after 24h. During the first eight days (representing at least one and at most two generations in all the treatments and species), the total numbers of nematodes were counted daily. After this period, the counting was conducted at day 10 and afterwards each $5^{th}$ day until day 35. On the $15^{th}$ and the $25^{th}$ day the entire population was transferred to a larger Petri dish (resp. 8 cm i.d. and 15 cm i.d.) with new agar medium (resp. 8mL and 12mL) and food availability was increased proportionally to the agar surface. The parental generation was not removed in this experiment in order to compare the treatments with the competition treatments of De Meester, et al. (2011; chapter II). Frozen-and-thawed *Escherichia coli* (50 µL of a suspension with a density of $3x10^{10}$ cells $ml^{-1}$) was used as a food source and added every tenth day. The difference in food concentration between this experiment and the temperature experiment may not play a significant role in the first seven days as shown for Pm I in dos Santos, et al. (2008). Nevertheless due to these differences in food concentration and the differences in set-up and in timing (which may result in different conditions of the stock cultures), comparisons of any of the studied parameters between the experiments cannot be

made and both experiments were analysed separately. To test if differences in set-up led to differences in our parameters, we compared the two overlapping treatments (treatment 20 °C in the temperature experiments (all conducted at a salinity of 25) and the treatment with a salinity of 25 in the salinity experiments (all conducted at 20°C)) from both experiments.

**Life history characteristics and statistical analyses**

We used parametric tests (2-way ANOVA/ 1-way ANOVA or t-test) where possible because they are more powerful compared to their non-parametric analogues (resp. PERMANOVA/ Kruskal-Wallis or Wilcoxon-rank test). When data were not normally distributed and/or variances were not homogeneous, we first transformed the data to be able to conduct a parametric test. Only when assumptions of parametric testing were still not met, we used non-parametric tests. All statistical analyses were conducted in R (R Development Core Team, 2008), except for the PERMANOVAs (performed in PRIMER (Anderson, 2001; Clarke & Gorley, 2006)). An overview of all statistical analyses and life history characteristics can be found in Table 1. More details about the design of the tests and post-hoc tests can be found in the following paragraphs.

*A. Minimum juvenile development time*

Because reproductive strategy varied from oviparous to viviparous depending on cryptic species and environmental conditions, we determined minimum juvenile development time rather than minimum development time. Minimum juvenile development time was taken as the time from the occurrence of the first juveniles till the appearance of the first new adults. Minimum juvenile development time was analysed using two-way ANOVA's to test for differences between the different temperatures on the one hand and between the different salinities on the other hand. For the salinity experiments, a log transformation was performed to achieve normality of the data. One-way ANOVA's (for temperature) and t-tests (for salinity) within species were also conducted to see the effect of the abiotic variable on the juvenile development time within individual species. In the temperature and salinity experiments, a non-parametric test (Kruskal-Wallis test for the temperature experiment and Wilcoxon rank-sum test for the salinity experiment) was used for the data of Pm I. A Tukey Honest Significant Differences test was conducted to see the pairwise significant differences for a significant factor. To test if the differences in set-up and timing of the two experiments had an effect on the results, a Wilcoxon rank-sum test was conducted between the two overlapping treatments.

*B. Instantaneous fecundity*

Numbers of juveniles and eggs produced after 24 h were divided by the number of inoculated adult females (4 for the temperature experiment and 5 for the salinity experiment) to obtain an estimate of per capita instantaneous fecundity. The influences of an abiotic factor (resp. salinity or temperature) and species identity were tested on the total number of offspring (sum of number of eggs and juveniles). To test for effects on reproductive strategy (ovopary/vivipary), separate analyses on the numbers of eggs and juveniles were also conducted. For the temperature experiments, no normality of data was achieved and PERMANOVA (Permutational Based Multivariate Analysis of Variance on the basis of Euclidean distance with 999 permutations) was conducted. PERMDISP (Anderson, 2004) was performed to test the homogeneity of dispersions (distance to the median). For the salinity experiments, data for juveniles and total offspring were analysed by two-way ANOVAs; for the eggs a PERMANOVA on the basis of Euclidean distance with 999 permutations on the log-transformed data (to achieve homogeneity of variances) was necessary. In the temperature experiment, within-species analyses were conducted with a parametric one-way ANOVA to assess the effect of the abiotic variable on the instantaneous fecundity within one species. Non-parametric Kruskal-Wallis tests were used for numbers of juveniles, eggs and total numbers of Pm IV, number of eggs for Pm II, and juvenile and total numbers of Pm III. In the salinity experiments, t-tests were conducted for numbers of juveniles, eggs and total numbers for all species, with the exception for Pm IV eggs and juveniles and Pm II eggs, for which a non-parametric Wilcoxon rank-sum test was conducted. A Tukey Honest Significant Differences test was conducted to assess the pairwise significant differences within a significant factor for the parametric analyses; pairwise Wilcoxon rank-sum tests with Bonferroni correction were used for the non-parametric analyses. A Wilcoxon rank-sum test was also conducted between the two overlapping treatments.

*C. Total population development*

Total nematode numbers (adults + juveniles) over time were used to analyse population development: for the temperature experiment, data until 21 days were used; the salinity experiment had a duration of 35 days. The effect of temperature or salinity on the population growth curves (total number of adults over time) was analysed with Generalised Estimating Equation models in R (GEEGLM) (Zuur, et al., 2009), because counts on different time moments were not independent of each other. Number of nematodes were entered in the

model as dependent variable, temperature/salinity and species as independent factors and time as a covariable (continuous variable). Time repeated measurements were incorporated by adding information about the dependence of the data to the model in the 'id' argument. The correlation structure specified in the model was an autoregressive correlation, as counts at two close time moments will be more correlated than two which are further apart. A log-transformation on the count data was conducted for both experiments, and time was squared ($time^2$) and added to the model to account for the non-linearity of the data. Non-significant factors were removed from the model, until a good model was achieved. Models were evaluated by checking the residuals against the fitted values to be randomly scattered without showing any systematic pattern. Quasilikelihood under the Independence model Criterion (QIC) was used to select the most appropriate model (Pan, 2001). Pairwise comparisons were done by dummy coding in the models, followed by a Bonferroni correction. The script of the GEEGLM models can be found in Appendix S1. In addition, two two-way ANOVA's were conducted on the average number of nematodes over time to check the effect of species and abiotic factor on the average number of nematodes.

Table 1: overview of all statistical analyses for the two different experiments.

| | | | | | |
|---|---|---|---|---|---|
| | Species x abiotic factor | | | | |
| | **juvenile development time** | **Instantaneous fecundity** | | | **Population development** |
| | | **eggs** | **juveniles** | **total** | |
| **temperature** | 2-way ANOVA | 2-way PERMANOVA | 2-way PERMANOVA | 2-way PERMANOVA | GEEGLM |
| **salinity** | 2-way ANOVA (log-transformed) | 2-way PERMANOVA (log-transformed) | 2-way ANOVA | 2-way ANOVA | GEEGLM |
| | Within-species analysis | | | | |
| | **juvenile development time** | **Instantaneous fecundity** | | | |
| | | **eggs** | **juveniles** | **total** | |
| **temperature** | Pm II/ Pm III/ Pm IV: 1-way ANOVA<br>Pm I: Kruskal-Wallis test | Pm I/ Pm III: 1 -way ANOVA<br>Pm II/ Pm IV: Kruskal-Wallis | Pm I/ Pm II: Pm IV: &-way ANOVA<br>Pm III: Kruskal-Wallis test | Pm I/ Pm III: 1-way ANOVA<br>Pm II/ Pm IV: Kruskal-Wallis | |
| **salinity** | Pm II/ Pm III/ Pm IV: 1-way ANOVA<br>Pm I: Wilcoxon-rank test | Pm I/ Pm III: t-test<br>Pm II/ Pm IV: Wilcoxon-rank | Pm I/ Pm III/ Pm IV: t-test<br>Pm II: Wilcoxon-rank test | all: t-test | |

## Results

No differences in juvenile development time and reproductive output were found between the two overlapping treatments of the temperature and salinity experiments (all $p>0.05$), suggesting that the differences in set-up did not strongly impact these life history traits of all four cryptic species.

### Minimum juvenile development time

In the temperature experiment, minimum juvenile development time was influenced by both species identity ($F_{3,36}=15.93$, $p=0.0018$) and temperature ($F_{2,36}=18.12$, $p=3.60e^{-6}$). No interaction effect between species identity and temperature was found ($p>0.05$). Across all species treatments, a significantly longer juvenile development time was observed at the lowest temperature (15°C) (resp. 6.80 ± 1.33 days for 15°C, 4.63 ± 0.61 days for 20°C and 3.41 ± 0.69 days for 25°C, Fig. 1a). A decreasing juvenile development time with increasing temperature was observed within each species, but the within-species ANOVA only showed a significantly lower juvenile development time at the highest temperature compared with the lowest temperature for Pm III ($F_{2,9}=9.00$, $p=0.0071$), and not for the other species. Moreover, the juvenile development time over all temperature treatments was significantly shorter for Pm III (3.50 ± 0.78 days) compared to Pm I and Pm II (resp. 5.67 ± 1.44 days and 6.05 ± 1.03 days (Fig. 1a). The minimum juvenile development time was significantly affected by salinity ($F_{1,24}=5.07$, $p=0.034$) and was lower at a salinity of 15 (3.65 ± 0.63 days) than at a salinity of 25 (4.50 ± 0.66 days). Within-species ANOVA revealed that these differences were only significant in Pm IV ($F_{1,6}=9.80$, $p=0.020$) (Fig. 1b). In contrast with the temperature experiment, juvenile development time was not shorter for Pm III compared with the other species. This may be explained by the within-species ANOVA for the temperature experiment, which only showed a significantly lower juvenile development time at the highest temperature compared with the lowest temperature for Pm III ($F_{2,9}=9.00$ , $p=0.0071$), and no difference between the species was found at a temperature of 20°C (conditions of the salinity experiment).

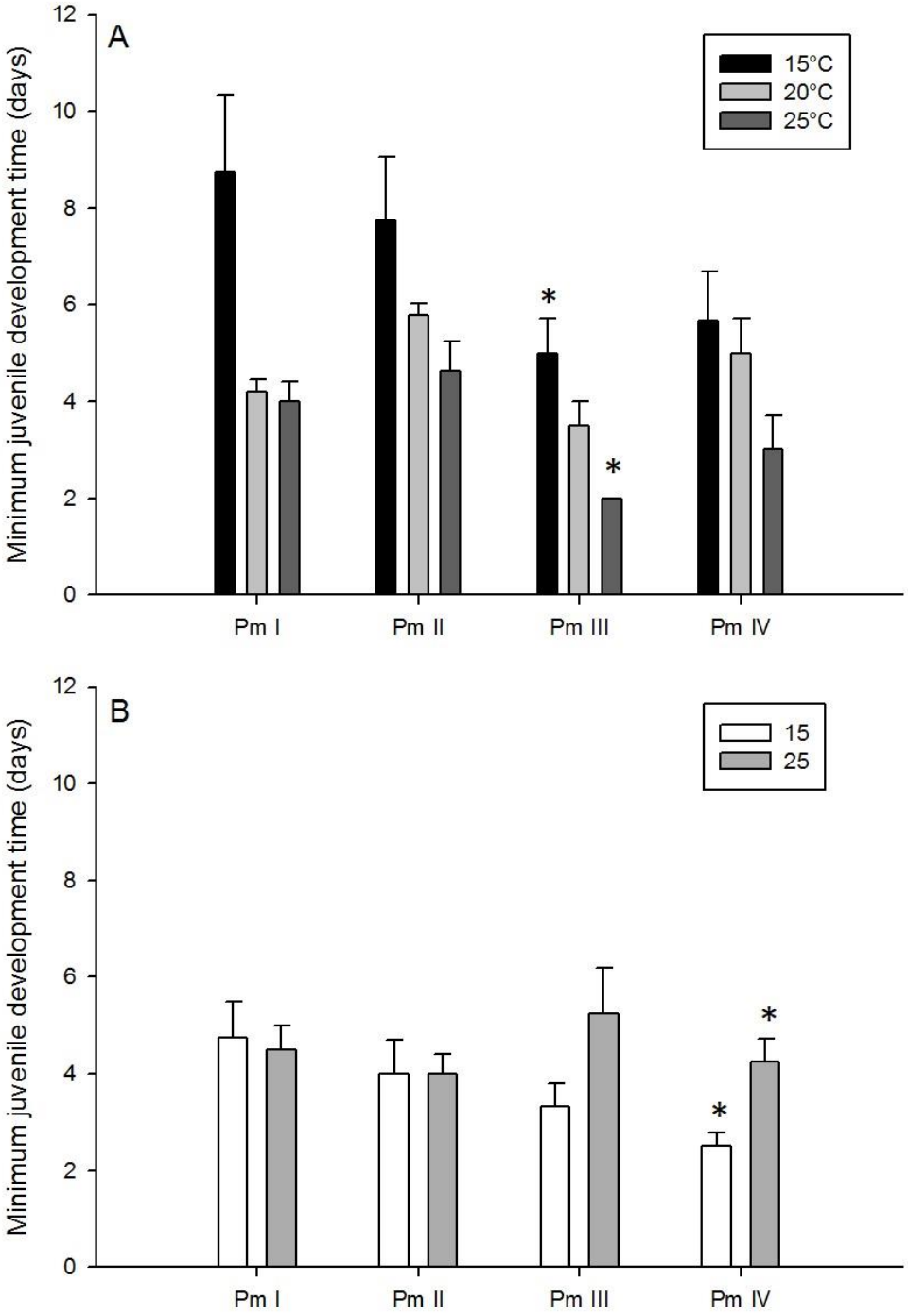



Figure 1: Minimum juvenile development time (mean ± SE) for the four cryptic species of *L. "marina"* at (a) three different temperature treatments (resp. 15°C, 20°C and 25°C) and (b) two different salinity treatments (resp. 15 and 25). Asterisks show pairwise significant values ($p<0.05$) within one species.

**Instantaneous fecundity**

In the temperature experiment, total instantaneous fecundity was significantly different between species ($F_{3,36}$=70.66, p=0.001), with a higher number of offspring for Pm III (7.06 ± 1.73) compared to Pm II and Pm IV (resp. 2.02 ± 0.98 and 1.98 ± 0.85). No effect of temperature or the interaction of temperature with species was found (all p>0.05). Within-species analyses showed that total number of offspring for Pm IV was lower at 15°C compared with 25°C ($W_{2,12}$=6.17, p=0.046). Species also showed differences in reproductive strategy (PERMANOVA on number of eggs: pseudo-$F_{3,36}$=36.40, p=0.001), with Pm II and Pm III being the only two species laying eggs (Fig. 2a). In the salinity experiment, total instantaneous fecundity was also influenced by species identity ($F_{3,24}$=6.30, p=0.003) but not by salinity. A higher number of offspring for Pm III (8.33 ± 1.90) was found compared with Pm I, Pm II and Pm IV (resp. 2.75 ± 0.94, 4.05 ± 1.35 and 4.63 ± 0.65). Within-species analyses also did not show any effect of salinity on total instantaneous fecundity (all p>0.05). However, Pm III produced more juveniles and less eggs at lower than at higher salinity, while Pm IV started laying eggs at a salinity of 15 (Fig. 2b).

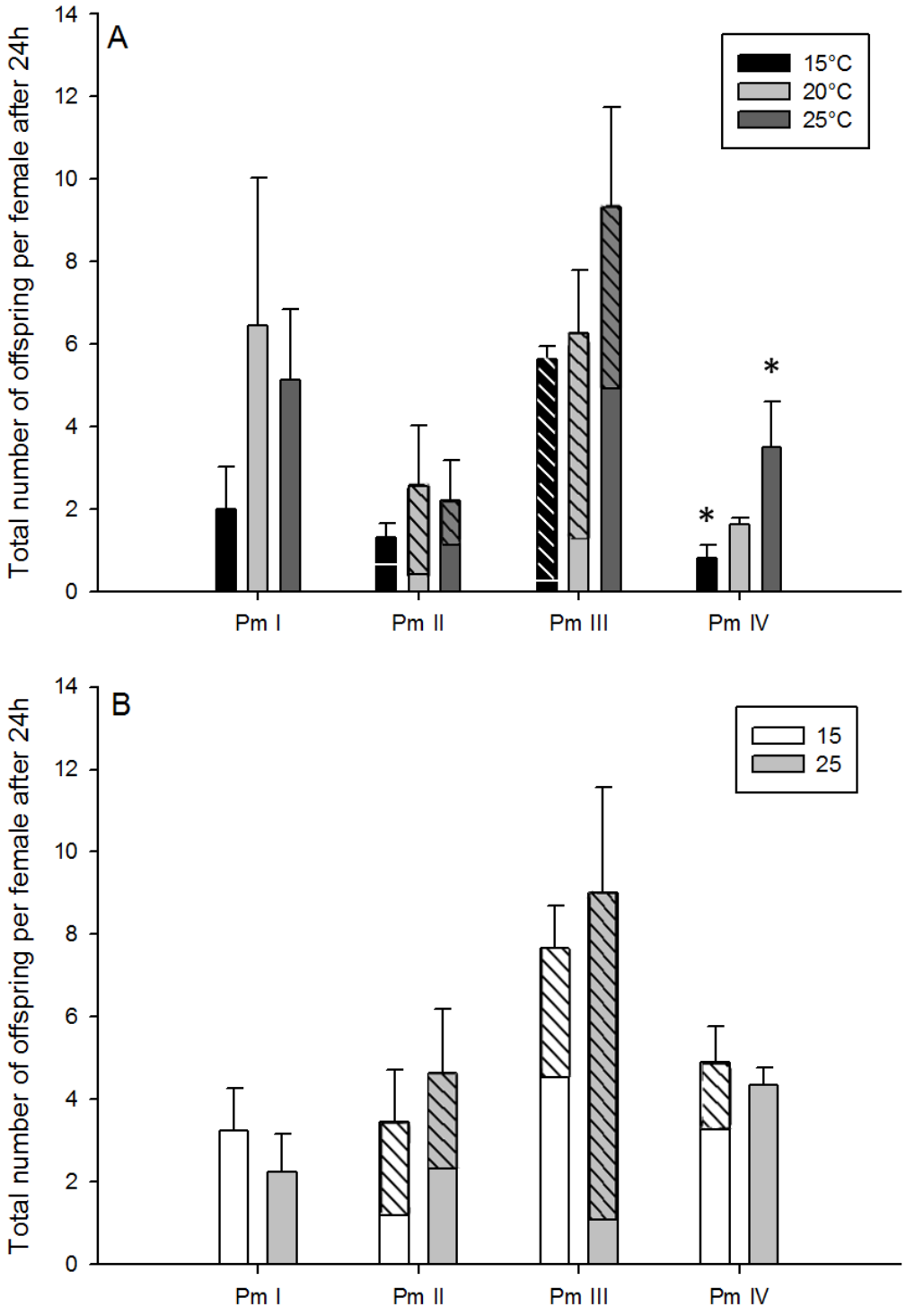



Figure 2: Instantaneous fecundity: total number of offspring per female after 24h (mean ± SE) for the four cryptic species of *L. "marina"* at (a) three different temperature treatments (resp. 15°C, 20°C and 25°C) and (b) two different salinity treatments (resp. 15 and 25). Striped parts of bars are the proportion of eggs in that specific treatment. Asterisks show pairwise significant values ($p<0.05$) within one species.

**Total population development**

The final models describing total population development in the temperature and salinity experiments can be found in Table 2. In the temperature experiment, total population development was influenced by the interaction between time, species and temperature (for significance levels, see Table 3). The increase of nematodes over time was species and temperature specific: growth curves differed for Pm III and Pm IV between all three temperatures. Pm III performed better at higher temperatures compared with the lower temperatures. At the start of the experiment, Pm IV also performed best at the highest temperature, but by the end of the experiment the highest nematode numbers were found at a temperature of 15°C. For Pm I, growth curves did not significantly differ between the temperatures, but very low numbers of nematodes at a temperature of 25°C were observed throughout the experiment. Finally, Pm II performed best at the lowest temperature (15°C) compared with the highest temperature (25°C, Fig. 3). At that high temperature, Pm III also performed better than Pm II. The two-way analysis on the time-averaged number of nematodes revealed a significant interaction between species and temperature ($F_{6,33}$= 2.92; p= 0.022), but the pairwise tests only showed differences between Pm II and Pm III (resp. 299 ± 34 and 27 ± 3 nematodes) at the lowest temperature.

In the salinity experiment, the interaction between time and species and the interaction between salinity and species influenced the population development (see Table 4). At the highest salinity, no differences in population growth were found between the species. At the lower salinity, Pm I and Pm II differed from Pm IV, due to the higher number of Pm IV nematodes (and not due to different growth curves, because no effect of the interaction of salinity with time was found) in the first 15 days at the lower salinity. Pm III also had higher abundances compared with Pm II. Pm III and Pm IV thus performed better at a salinity of 15 compared with a salinity of 25. This was also confirmed by a two-way ANOVA on time-averaged numbers, where Pm III and Pm IV had higher abundances at the lower compared with the higher salinity ($F_{3,22}$=10.72, p=0.001; a difference resp. of 250 ± 46 nematodes for Pm III and 173 ± 18 nematodes for Pm IV).

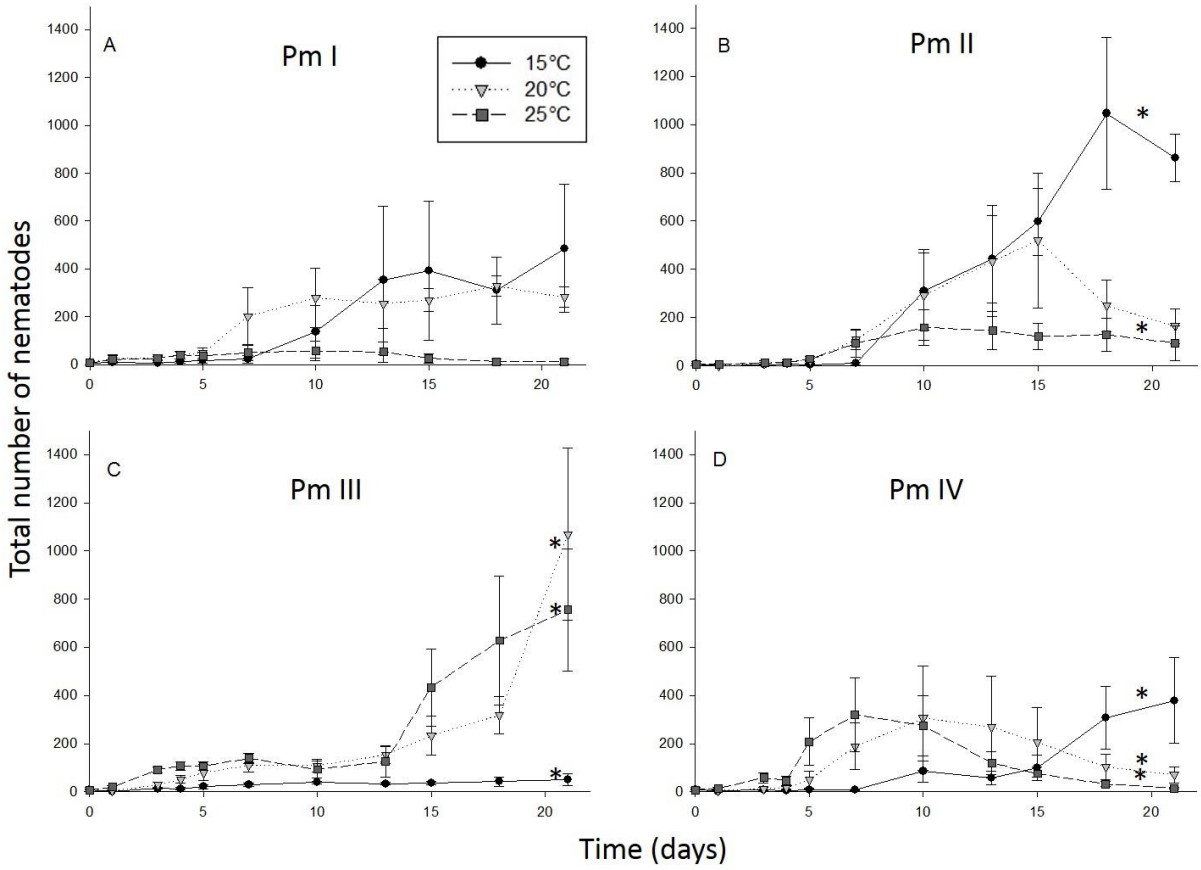



Figure 3: Population development over time (mean ± SE) at three different temperature treatments (resp. 15°C, 20°C and 25°C) for the four cryptic species of *L. "marina"*: (a) Pm I, (b) Pm II, (c) Pm III and (d) Pm IV. Asterisks above the curve show significant differences in population development between temperatures within one species.

Table 2: final GEEGLM models for the temperature and salinity experiment.

| | |
|---|---|
| **Temperature** | log(number of nematodes)~time + temperature + species + time x species + time x temperature + temperature x species + time x temperature x species + time$^2$ |
| **Salinity** | log(number of nematodes)~time + salinity + species + time x species + salinity x species |

Table 3: Results of the Wald Statistics of the GEEGLM: effect of time, temperature (15, 20 or 25°C) and species (Pm I, Pm II, Pm III and Pm IV) on the number of adults of *Litoditis "marina"*. Confidence level: 95%.

| | Df | $\chi^2$ | P |
|---|---|---|---|
| **Time** | 1 | 30.9 | **< 0.0001** |
| **Temperature** | 2 | 2.2 | 0.33 |
| **Species** | 3 | 1.3 | 0.74 |
| **Time$^2$** | 1 | 65.1 | **< 0.0001** |
| **Time:Temperature** | 2 | 22.0 | **< 0.0001** |
| **Time:Species** | 3 | 5.4 | 0.14 |
| **Temperature:Species** | 6 | 24.0 | **0.0005** |
| **Time:Temperature:Species** | 6 | 41.8 | **< 0.0001** |

Table 4: Results of the Wald Statistics of the GEEGLM: effect of time, salinity (15 or 25) and species (Pm I, Pm II, Pm III and Pm IV) on the number of adults of *Litoditis "marina"*. Confidence level: 95%.

| | Df | $\chi^2$ | P |
|---|---|---|---|
| **Time** | 1 | 1.0 | 0.32 |
| **Species** | 3 | 1.0 | 0.81 |
| **Salinity** | 1 | 1.9 | 0.17 |
| **Time:Species** | 3 | 68.1 | **< 0.0001** |
| **Salinity:Species** | 3 | 9.8 | **0.020** |

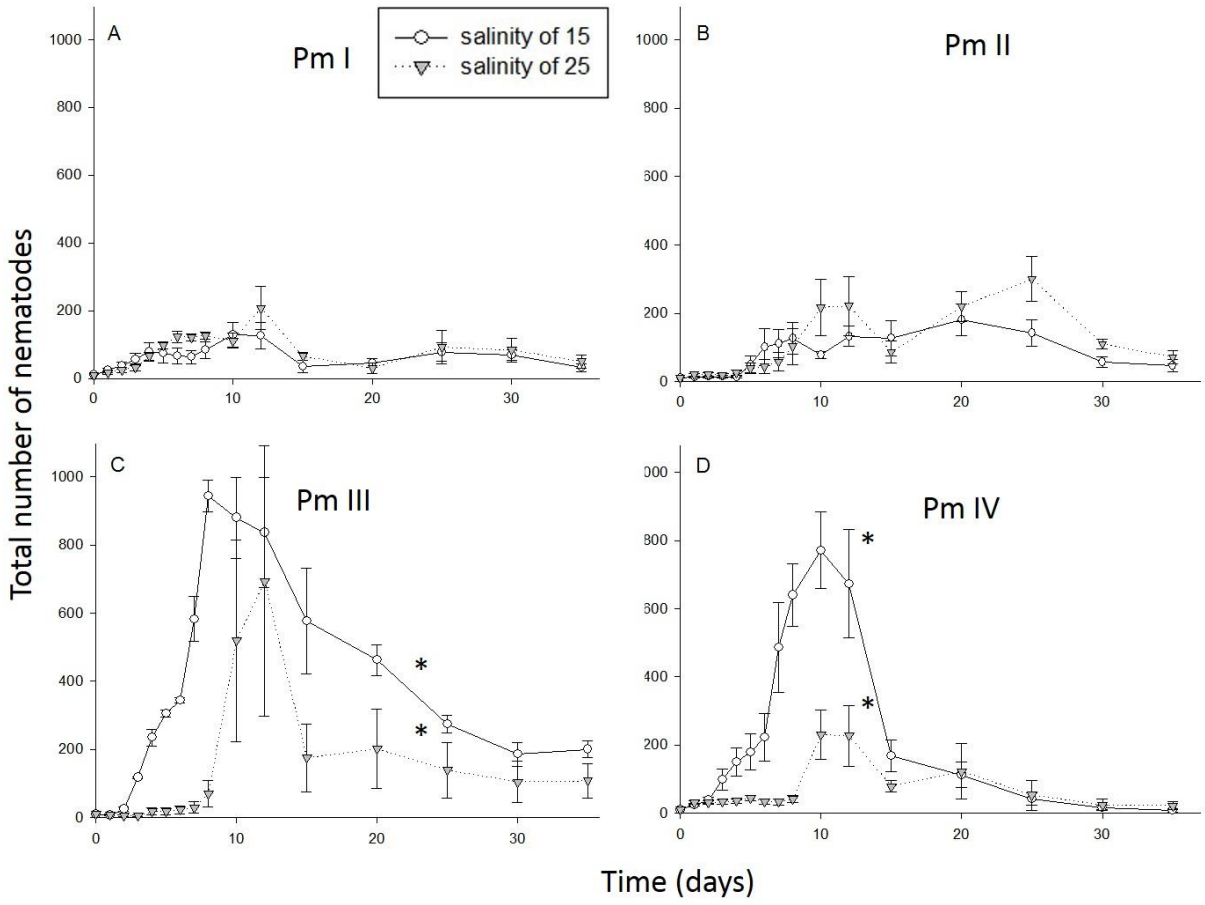



Figure 4: Population development over time (mean ± SE) at two different salinity treatments (resp. 15 and 25) for the four cryptic species of *L. "marina"*: (a) Pm I, (b) Pm II, (c) Pm III and (d) Pm IV. * above the curve shows significant differences in population development between salinities within one species.

## Discussion

Our results show that consistent differences in life history are present between the four morphologically (nearly) identical species of *L. "marina"*. Furthermore, abiotic factors affect the life history characters of some of these cryptic species (Table 5). Temperature clearly had a stronger effect on the investigated life-history characters than salinity: three of the four cryptic species were affected in their population growth by temperature, compared with only two for salinity.

Table 5: Overview of the significant differences in life history traits between cryptic species of *Litoditis "marina"* under different abiotic conditions.

| | **Minimum juvenile development time** | **Instantaneous fecundity** | **Population development** |
|---|---|---|---|
| SPECIES-SPECIFIC DIFFERENCES | Pm III < Pm I ; Pm II | Pm III > Pm I; Pm II; Pm IV<br>Vivipary: Pm I and Pm IV<br>Ovipary: Pm II and Pm III | No differences between the species at the stock conditions (salinity of 25, 20°C) |
| TEMPERATURE | 15°C > 25°C<br>Pm III: 15°C > 25°C | Pm IV: 15°C < 25°C | Pm III ; Pm IV: 25°C >20°C >15°C<br>Pm II: 15°C > 25°C<br>25°C: Pm III > Pm II |
| SALINITY | Pm IV: 15 > 25 | 15: Pm III juveniles ↑<br>Pm IV eggs ↑<br>Total reproduction =<br>Pm III: 15 > 25 | 15: Pm I; Pm II < Pm III ; Pm IV<br>Pm III: 15 > 25<br>Pm IV: 15 > 25 |

**Species-specific differences in life histories between the cryptic species exist regardless abiotic conditions**

Substantial differences in life history between the four cryptic species of *Litoditis "marina"* exist, regardless the abiotic conditions. Pm III had a higher instantaneous fecundity than the other three species. Phylogenetic analyses of three molecular loci (COI, ITS, D2D3) showed that Pm III is most distantly related to the three other species (Derycke, et al., 2008a). Moreover, Pm III is also morphologically more distinct when combining different morphological traits (Fonseca, et al., 2008). Our results show that these differences are also reflected in life history traits. The higher fecundity in conjunction with its comparatively good active dispersal capacity (De Meester, et al., 2012; chapter VI) suggest that this species may be a superior colonizer compared to the other three cryptic species.

Differences in reproduction strategy were observed between the cryptic species: Pm II and Pm III were always oviparous in this experiment, while Pm I and Pm IV were mostly viviparous species (except for Pm IV under certain abiotic conditions, see further.). Oviparous females deposit eggs that develop and hatch in the external environment. Viviparous females retain developing eggs inside their reproductive tracts and give birth to free-living offspring. The advantage of the latter is that embryos are protected by the pregnant female; the disadvantage is a more limited reproductive output. Vivipary may particularly occur in harsh environments, because the body may protect progeny against environmental stress (Blackburn, 1999). Pm I and Pm IV were mostly viviparous in our test conditions, but bagging or endotokia matricida was also frequently observed. Bagging, i.e. egg retention with internal hatch causing maternal death, is seen as a change from ovipary to vivipary in *Caenorhabditis elegans* (Chen & Caswell-Chen, 2004). Bagging is a plastic life history trait, which happens under a range of stressful conditions, such as overcrowding, and it is inducible. Moreover, reproductive strategies may be differentially influenced by abiotic conditions (Chen, Caswell-Chen, 2004; Wear, et al., 1986), as seen for Pm IV: this species changed its reproductive output when salinity changed (see further). We can assume that a species-specific preferred strategy exists, in which the costs and gains of a specific reproductive strategy differ between the species.

**Temperature had a stronger effect on life history characteristics than salinity**

Temperature had a substantial effect on the life history traits of all the cryptic species. The juvenile development time increased at lower temperatures. Within the temperature limits of an organism, an increasing temperature mostly results in a faster development due to the

faster enzymatic reactions (Wear, et al., 1986; Savage, et al., 2004). Faster development at higher temperatures had already previously been documented for *Litoditis "marina"*, but in none of these studies the identity of the cryptic species was known (e.g. Tietjen & Lee, 1972; Hopper, et al., 1973; Gaudy, et al., 1982; Moens & Vincx, 2000b). The effect of temperature on juvenile development time was most pronounced for Pm III. In addition to a shorter juvenile development time at the highest temperature, Pm III (and Pm IV) also produced a higher number of juveniles at the highest temperature. This was reflected in the population development of Pm III, which performed better at the highest temperature over the whole experiment. Pm IV also showed a faster population development at the highest temperature, but only so at the start of the experiment. After 21 days, Pm IV was more abundant at the lowest temperature, indicating that the higher initial reproduction at higher temperature may be the result of a short-term stress response to a sudden change in environmental conditions (Grainger, 1958) from the stock conditions (salinity of 25 , 20°C), which was also observed at the lower salinity (De Meester, et al., 2011; chapter II). Another possibility is that intraspecific competition becomes too high and resulted in lower reproduction. Pm II performed best at the lowest temperature, despite a higher juvenile development time and no significant differences in its instantaneous reproductive output compared with the other temperatures. This may indicate a higher survival rate at lower temperature or an increased reproductive output after the first days. In contrast to the three other species, temperature did not show any effects on the life history traits of Pm I, suggesting that Pm I is a more eurytherm species. A eurythermic strategy may be advantageous for species in heterogeneous environments (e.g. temporal and/or tidal environments) in which abiotic conditions fluctuate strongly (Van Tienderen, 1991; Gilchrist, 1995), and may explain why Pm I was the most dominant species in littoral habitats along the Belgian and southeast Dutch coast and estuaries throughout the year, whereas Pm II, Pm III and Pm IV showed more pronounced seasonal fluctuations (Derycke, et al. 2006, see further).

*Litoditis "marina"* has been suggested to be a euryhaline species, with limited effects of salinity on its life cycle (Tietjen, et al., 1970). Nevertheless, its juvenile development time was shorter at the lower salinity for all cryptic species, which was most reflected in Pm III and Pm IV. Moreover, population development of Pm III and Pm IV was clearly affected by salinity. Pm III reached a higher abundance at lower salinity throughout the whole experiment. Nevertheless, Pm III did not produce more offspring at the lower salinity, but more juveniles and less eggs occurred compared with the higher salinity, indicating a partial

shift to vivipary or a faster egg hatch. The higher number of nematodes may be explained by a higher survival at the lower salinity. Pm IV clearly started to lay more eggs at the lower salinity, or held the eggs for a shorter time period inside the body. Nevertheless, they did not produce more offspring. Numbers of nematodes were, as in Pm III, higher at lower salinity, but the differences in population development between the two salinities disappeared after 12 days, indicating that Pm IV probably shows a stress response at the start of the experiment (De Meester, et al., 2011; chapter II).

**Differential abiotic preferences can help to explain coexistence between cryptic species**

Pm I, Pm II and Pm III have been found together in several locations in the Westerschelde, with different relative abundances throughout the year; Pm IV co-occurred with Pm II and Pm III in a marine lake, again with seasonally variable relative abundances (Derycke, et al., 2006). The fact that assemblage composition differs among locations and seasons suggests that abiotic conditions may be important to explain the co-occurrence of these cryptic species. Previous research has shown that Pm I is abundant throughout the year (Derycke, et al., 2006), which nicely agrees with its eurytherm behaviour in the temperature experiment. Based on our results, we could expect Pm II and Pm IV to perform comparatively better at lower temperatures and indeed, in natural conditions these species were least abundant during summer (Derycke, et al., 2006). Pm II was also abundant in the Baltic Sea, in which the temperature during the year is lower than in the North Sea, which is also in agreement with our results. Moreover, Pm III was most abundant in summer, in line with its preference for higher temperatures in the present experiment. Temperature may thus be an important factor influencing coexistence and population dynamics under natural conditions. The effect of salinity was not clearly reflected in the geographical distributions, which may be due to the small salinity range studied here. Pm IV dominates at lake Grevelingen, where the average salinity exceeds 30, but was not found at locations with a lower salinity, though its population development was better at the lower salinity in our experiment. Pm II dominates in the Baltic Sea, in which the salinity is not higher than 15, but did not perform better at this salinity in our experiment. Moreover, Pm III distributions could not be correlated easily with its preference for the lower salinity. These results indicate that in the limited range of salinities and temperatures tested in our experiment, temperature is a more important factor influencing niche differentiation than salinity, in agreement with a recent study on another coastal cryptic nematode species complex (Van Campenhout, et al., 2014). In contrast, daily

temperature fluctuations in the same range (from a night temperature of 15°C to a day temperature of 25°C) did not cause significant differences in population performance between the cryptic species of *L. "marina"* (De Meester, et al., 2015c; chapter IV). This diurnal variation was not incorporated in the present experiments, but it may also be important in natural situations. Next to abiotic conditions, other factors (biotic factors, dispersal rates,…) may influence the geographic distributions of the cryptic species. Interspecific competition has an influence on abundances of the *L. "marina"* cryptic species (De Meester, et al., 2011; chapter II). For instance, Pm IV was a poor competitor in closed microcosms without habitat structure, and this could explain why Pm IV has not been found in field surveys except at a salinity of more than 25 (Derycke, et al., 2006), where it may be able to avoid being outcompeted by the other species. Furthermore, Pm IV was mostly found on thalli of *Ulva* sp. while the other species have been isolated from *Fucus* sp., which may indicate different preferential habitats between the species. In addition, interspecific interactions change when abiotic factors change (De Meester, et al., 2011 (chapter II); 2015c (chapter IV), which may have important repercussions for coexistence. Moreover, differences in dispersal rates between the species also have an influence on community dynamics, at least in laboratory trials (De Meester, et al., 2012 (chapter VI); 2015b (chapter VII)). In the present study, the role of the environment has been examined by experimental variation of single isolated factors. It is evident that in nature many factors act simultaneously. Temperature and salinity are among the most important physical factors in the life of marine organisms and there is often a complex co-relationship between these two factors, where temperature can modify the effects of salinity and vice versa (Wear, et al., 1986). It is nevertheless promising to find such a good match between single-factor responses (here temperature) and seasonal patterns found in the field (Derycke, et al., 2006).

**Conclusions**

Despite the high morphological similarity, cryptic species of *Litoditis "marina"* show differences in life history traits and differential reproductive strategies. Moreover, life history traits are influenced by abiotic conditions in a species-specific way. This may have important consequences for interspecific interactions. Different preferences of the cryptic species in one abiotic factor may already result in niche differentiation, which may lead to coexistence of closely related species.

## Acknowledgements

Funding for this research was obtained from the Flemish Fund for Scientific Research (FWO) through project G038715N, and from Ghent University through projects 011060002 and 01GA1911W. Nick De Meyst is acknowledged for the set-up and counts of the temperature experiment. This research has benefitted from a statistical consult with the Ghent University FIRE (Fostering Innovative Research based on Evidence) service.

## Supplementary information

**Appendix S1:** R script for GEEGLM on population development (temperature experiment)

```
library(geepack)
## population development temperature experiment
# importing data
temperature = read.table("popdev_temp.txt", header = T)
temperature$time2=temperature$time^2
log.adults=log(adults+1)
temperature=data.frame(temperature, log.adults)

#creating model
M1A <- geeglm(log.adults~time*temp*spec, data = temperature, id = rep, corstr = "ar1")
anova(M1A)
summary(M1A)
plot(fitted(M1A), residuals(M1A))
abline(h=0, col='red') # look for random scatter

M1B <- geeglm(log.adults~time*temp*spec+time2, data = temperature, id = rep, corstr = "ar1")
anova(M1B)
summary(M1B)
plot(fitted(M1B), residuals(M1B))
```

```
abline(h=0, col='red') # look for random scatter


M1B2 <- geeglm(log.adults~time*temp*spec+time2*temp*spec, data = temperature, id =
rep, corstr = "ar1")
anova(M1B,M1B2, test="LRT")
summary(M1B)
plot(fitted(M1B2), residuals(M1B2))
abline(h=0, col='red') # look for random scatter


##Comparing models: QIC
QIC = function(model.R) {
 library(MASS)
 model.indep = update(model.R, corstr = "independence")
 # Quasilikelihood
 mu.R = model.R$fitted.values
 y = model.R$y
 type = family(model.R)$family
 quasi.R = switch(type,
                  poisson = sum((y*log(mu.R)) - mu.R),
                  gaussian = sum(((y - mu.R)^2)/-2),
                  binomial = sum(y*log(mu.R/(1 - mu.R)) + log(1 - mu.R)),
                  Gamma = sum(-y/mu.R - log(mu.R)),
stop("Error: distribution not recognized"))}


sapply(list(M1B, M1A), QIC)
```

**#Backwards step selection procedure if necessary**

**## Some examples for pairwise comparisons with dummy coding**

# create a specific linear combination of components of beta

# check that X*beta is the first component only

# note that matrix multiplication is %*%, not *

Summary(M1B)

##Pm1H vs Pm1L

X<-c(0,0,1,0,0,0,0,0,1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0)

esticon(M1B, X)

##Pm1M vs Pm1L

X1<-c(0,0,1,-1,0,0,0,0,1,-1,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0)

esticon(M1B, X1)

##Pm2H vs Pm2M

X2<-c(0,0,0,1,0,0,0,0,0,1,0,0,0,0,1,0,0,0,0,0,1,0,0,0,0)

esticon(M1B, X2)

##Pm2H vs Pm2L

X3<-c(0,0,1,0,0,0,0,0,1,0,0,0,0,1,0,0,0,0,0,1,0,0,0,0,0)

esticon(M1B, X3)

#Perform a Bonferroni correction

# CHAPTER IV


# DAILY TEMPERATURE FLUCTUATIONS ALTER INTERACTIONS AMONG CLOSELY RELATED SPECIES

---

Slightly modified from:

De Meester, N., dos Santos, G.A.P., Rigaux, A., Valdes, Y., Derycke, S. & Moens, T. (2015) Daily temperature fluctuations alter interactions between closely related species of marine nematodes. *PloS one*, 10, e0131625

## Abstract

In addition to an increase in mean temperature, climate change models predict decreasing amplitudes of daily temperature fluctuations. In temperate regions, where daily and seasonal fluctuations are prominent, such decreases in daily temperature fluctuations can have a pronounced effect on the population performance of species and on the outcome of species interactions. In this study, the effect of a temperature regime with daily fluctuations versus a constant temperature on the population performance and interspecific interactions of three cryptic species of the marine nematode species complex of *Litoditis "marina"* (Pm I, Pm III and Pm IV) were investigated. In a lab experiment, different combinations of species (monospecific treatment: Pm I and Pm IV and Pm III alone; two-species treatment: Pm I + Pm IV; three-species treatment: Pm I + Pm IV + Pm III) were subjected to two different temperature regimes: one constant and one fluctuating temperature. Our results showed that fluctuating temperature had minor or no effects on the population performance of the three species in monocultures. In contrast, interspecific interactions clearly influenced the performance of all three species, both positively and negatively. Temperature regime did have a substantial effect on the interactions between the species. In the two-species treatment, temperature regime altered the interaction from a sort of mutualism to commensalism. In addition, the strength of the interspecific interactions changed depending on the temperature regime in the three-species treatment. This experiment confirms that interactions between the species can change depending on the abiotic environment; these results show that it is important to incorporate the effect of fluctuations on interspecific interactions to predict the effect of climate change on biodiversity.

## Introduction

Temperature is one of the most important environmental factors affecting many aspects of the life cycles of species (e.g. development and growth rates, body size, reproduction, etc.), and is considered an important selective agent (Cossins & Bowler, 1987). Over the past 100 years, global temperature has increased by approximately 0.6 C (Marcott, et al., 2013).

Climate change models not only predict rising average temperatures, but also an increasing frequency of episodic temperature extremes (Easterling, et al., 2000) and decreasing amplitudes in daily temperature fluctuations (Walther, et al., 2002). In temperate regions, where daily and seasonal fluctuations are prominent, such decreases in daily temperature fluctuations can have a pronounced effect on the population performance of species as well as on the outcome of species interactions. For instance, lower maximum peak temperatures can have species-specific effects on development rate, survival and reproduction of individual species. Such effects are difficult to predict, since both increased and decreased development rates have been observed under a fluctuating compared to a constant temperature regime (e.g. Brakefield & Kesbeke, 1997; Pétavy, et al., 2001). In addition, a higher mortality and lower reproduction rate at constant temperature have frequently been found (e.g. Johnson & Shick, 1977; Colinet, et al., 2007). Species-specific responses to daily fluctuations can potentially influence species interactions in three ways: directly, by changing the competitive abilities of species; indirectly, as a result of changes in population dynamics of one of the species which indirectly influences other species (e.g. by food depletion); or by a combination of both direct and indirect processes (Litchman & Klausmeier, 2001). Moreover, if species respond differentially to environmental fluctuations, daily temperature cycles can contribute to a stable coexistence between species (Descamps-Julien & Gonzalez, 2005). Closely related species are expected to have high competition (Darwin, 1859), and changes in temperature fluctuations may therefore lead to changes in interspecific interactions and facilitate the co-occurrence of species.

An intriguing case of coexistence is that of closely related, morphologically highly similar cryptic species. These cryptic species are morphologically indistinguishable, but genetically different (Bickford, et al., 2007). Coexistence of cryptic species in natural environments has been reported at small geographical scales in a broad range of taxa (e.g. Molbo, et al., 2003; Zhang, et al., 2004; Derycke, et al., 2008a; Peng, et al., 2008), and interactions between cryptic species have been commonly observed (e.g. Ortells, et al., 2003; De Meester, et al., 2011 (chapter II)). Despite this natural coexistence, some laboratory studies have shown that

permanent coexistence between closely related species is unlikely under constant environmental conditions (Hanski & Ranta, 1983; De Meester, et al., 2011 (chapter II)). If species have different abiotic optima, environmental fluctuations may be important in maintaining coexistence, and as a consequence, decreasing amplitudes of daily environmental fluctuations may affect the coexistence of cryptic species.

Cryptic diversity has been frequently observed in coastal nematodes (Derycke, et al., 2013). In the morphospecies *Litoditis "marina"* (Sudhaus, 2011); henceforth referred to as *L. "marina"*, formerly known as *Rhabditis marina* or *Pellioditis marina*, at least 10 cryptic species have been found (Derycke, et al., 2008b). Species of the *L. "marina"* species complex are typical colonizers of decaying algae and show explosive population growth and rapid colonization/extinction dynamics (Derycke, et al., 2007a). These species show concordant molecular divergences at nuclear and mitochondrial loci (COI, ITS, D2D3), but lack single distinctive morphological differences (Derycke, et al., 2008a; 2008b; Fonseca, et al., 2008). Four of them (Pm I, Pm II, Pm III and Pm IV) frequently occur in the littoral zone of the south-western coast and estuaries of The Netherlands (Derycke, et al., 2006; 2008b), in which pronounced daily temperature fluctuations are common. Pm I and Pm IV are the most closely related species but cross-breeding between them does not occur (Fonseca, et al., 2008). Sympatric occurrence of two or more of these species on decomposing algae is rule rather than exception (Derycke, et al., 2006; 2008b). This coexistence is intriguing since competition between the species exists (De Meester, et al., 2011; chapter II): Pm I and Pm III proved to be competitively superior to Pm II and Pm IV, but the precise nature of this competition is still unknown, and it may shift from contest to scramble competition depending, among other things, on the abiotic environment. Moreover, facilitation has been demonstrated between these four cryptic species of *Litoditis marina* in experiments using closed microcosms (De Meester, et al., 2011; chapter II). Dispersal may be one of the mechanisms enabling temporary coexistence (De Meester, et al., 2015b). Moreover, differential population responses to salinity (De Meester, et al., 2015a; chapter III) and partial differences in their gut bacterial communities (Derycke, et al., 2016; chapter V) suggest at least some degree of niche differentiation. Moreover, species-specific responses to temperature in a range of 15 to 25 °C exist (De Meester, et al., 2015a; chapter III). Salinity can influence these interspecific interactions (De Meester, et al., 2011; chapter II), but the effect of temperature on these interspecific interactions has not been investigated.

In this study, the effect of a temperature regime with daily fluctuations versus a constant temperature on (a) the population performance (here estimated from population size, both for juveniles and adults, (Benton & Grant, 2000)) and (b) interspecific interactions of three cryptic species of the *L. "marina"* species complex (Pm I, Pm III and Pm IV) were investigated. Based on previous research (Derycke, et al., 2008b; De Meester, et al., 2011 (chapter II); 2015a (chapter III)), we expected that (a) species which perform better at higher temperatures may, due to the higher maximal temperature in the fluctuating temperature regime, also have a higher population performance at fluctuating temperature. Pm III showed a geographical distribution in warmer regions compared with the other species (Derycke, et al., 2008b) and had a shorter juvenile development time at higher temperature (De Meester, et al., 2015a; chapter III), which suggests that Pm III may have a higher population performance at fluctuating temperature, due to the higher maximal temperature in this regime. This can also affect species interactions (b), with a dominance of the species with a higher population performance. We can thus expect that Pm III will be dominant over Pm I and Pm IV. However, differences in competitive abilities between the different species can also affect these interactions and temperature fluctuations can have indirect species-specific effects on them. Pm I was found to be competitively superior to Pm IV (De Meester, et al., 2011; chapter II), so we expected Pm IV to have very low abundances or even to go extinct. This study can help us to better understand the coexistence of these closely related species on small spatial scales in natural environments.

## Materials & Methods

### Nematode cultures

Nematodes for the experiments were harvested from monospecific stock cultures in exponential growth phase. Monospecific cultures of three different cryptic species (Pm I, Pm III and Pm IV) were each raised from one single gravid female, obtained from the field (for Pm I and Pm III Paulina marsh, Westerschelde, The Netherlands; for Pm IV Lake Grevelingen, The Netherlands) in September 2009, and maintained on sloppy (1%) nutrient:bacto agar media (temperature of 20°C; salinity of 25) with unidentified bacteria from their habitat as food (Moens & Vincx, 1998). The temperature of the stock cultures is comparable with the average temperature in the field during summer, while a salinity of 25 approximates the mean salinity in their natural environment.

### Temperature experiments

The experiment comprised three monospecific treatments (respectively M1, M3 and M4 for Pm I, Pm III and Pm IV), one two-species treatment with the two most closely related species: Pm I and Pm IV (D), and one treatment with all three species (T). These treatments (M, D or T) were called the 'interspecific interaction' treatment. Three females and two males per cryptic species were incubated in all treatments using an additive design (Jolliffe, 2000). Hence, total number of nematodes and species varied depending on the treatment (5 nematodes for M, 10 for D and 15 for T). Because intraspecific competition is known to be prominent in these nematodes (De Meester, et al., 2015b; chapter VII), numbers per species were kept constant in order to be able to elucidate the effect of interspecific competition in all treatments. By adjusting the size of the petri dish, the amount of agar medium and the amount of food (see further), the available space and resources per inoculated nematode in every treatment were kept constant. Monospecific treatments (M1, M3 and M4) were incubated in small petri dishes (inner diameter of 5.4 cm) with 4 mL of 1% bacto agar and 50 µL of a suspension of frozen-and-thawed *Escherichia coli* (strain K12, density of $3x10^{10}$ cells $mL^{-1}$ (dos Santos, et al., 2008)). The D treatment was incubated on petri dishes with the same inner diameter of 5.4 cm, but with 8 mL of 1% bacto agar medium and 100 µL of the same *E. coli* suspension used for the monospecific treatments. Finally, treatment T contained the three species together on petri dishes with an inner diameter of 8.4 cm, 12 mL of 1% bacto agar and 150 µL of *E. coli* suspension. Food was added at the start of the experiment and again after 14 days. Each treatment was replicated nine times (3 replicas at 3 time moments) at two different temperature regimes: a constant air temperature (C) of 20°C and

a fluctuating air temperature (F) with 12h of 15°C followed by 12h of 25°C (the change in temperature took approximately half an hour to establish and stabilize). A temperature of 20°C represents average summer temperature, whereas 15°C and 25°C represent fairly common daytime minimum and maximum temperatures during summer (Vlaams-Nederlandse Scheldecommissie, Werkgroep Onderzoek en Monitoring). The average temperature was equal in both treatments. All plates were sealed with Parafilm, which prevents evaporation of the agar but still allows oxygen diffusion into the plates. Salinity of the agar medium was 25. The pH of the agar medium was buffered at 7.5 – 8 with TRIS-HCl in a final concentration of 5mM, which increases the initial salinity by ca. 1.2 units. Cholesterol (100 µL $L^{-1}$) was added as a source of sterols, because nematodes on a purely bacterial diet appear incapable of *de novo* synthesis of specific sterols (Vanfleteren, 1980). After 7, 14 and 21 days, three replicates of every treatment (temperature regime x 'interspecific interaction' treatment) were frozen (- 20°C) for later counts and analysis of the assemblage structure and abundance. For the counts of adults and juveniles we used a stereomicroscope for all treatments. Relative quantification of each species in the D and T treatments was based on DNA extraction and qPCR analysis (Derycke, et al., 2012) following the same method as in a previous paper (De Meester, et al., 2015b; chapter VII). Absolute numbers per species were calculated by multiplying the relative abundances with the total numbers of the plate.

**Statistical analyses**

*A. Effect of temperature on the population performance of the species*

Within each species, 3-way ANOVAs were conducted on the numbers of adults and juveniles separately to test the effect of temperature (C or F), interspecific interactions (in case of Pm I and Pm IV: M1/M4, D and T and in case of Pm III: M3 and T) and time. No overall ANOVA with species as factor could be conducted as the data of the different species are not independent from each other within the D and T treatment. ANOVAs were conducted in the statistical software package R (R Development Core Team, 2008). A Tukey Honest Significant Differences test was performed on the highest significant factor. To achieve normality of the data, a log transformation was performed for data of adults and juveniles of Pm I and for juveniles of Pm IV. For Pm III adults, a PERMANOVA (Anderson, 2001)(on the basis of Euclidean distance with 999 permutations) was conducted because the assumptions for normality were not met, even after transformation. A pairwise

PERMANOVA was conducted on the significant factors. PERMDISP was performed to test the homogeneity of variances (distances to the median).

*B. Effect of fluctuating temperature on the interactions between the species*

PERMANOVA was also used to investigate the effect of the different temperature regimes, interspecific interactions and time on juvenile and adult assemblage dynamics. This was done by comparing adult and juvenile assemblage compositions in fictitious and real assemblages. A fictitious assemblage (F) per time moment and treatment was constructed by using the relative abundances of each species in the monospecific treatments (respectively M1 + M4 (=FiD) and M1 + M3 + M4 (=FiT)). These fictitious assemblages reflect species composition without interspecific interactions and were compared with the assemblage compositions in which more than one species was present and interspecific interactions were possible (FiD vs. D and FiT vs. T). The relative contribution of each species was the dependent variable, and the independent fixed factors were time (day 7, 14 and 21), temperature regime (C or F) and interspecific interactions (for Pm I and Pm IV: FiD (no interspecific interactions) compared with D (with interspecific interactions), for Pm I, Pm III and Pm IV: FiT (no interspecific interactions) compared with T (with interspecific interactions)). Significant terms and interactions were investigated using posterior pair wise comparisons within PERMANOVA. PERMDISP was performed to test the homogeneity of multivariate dispersions (distance to the centroid). A log transformation on the adults was used for the treatment with two species and a fourth root transformation on the juveniles was performed in the treatment with three species to achieve this homogeneity. A SIMPER analysis was used to identify which species primarily accounted for the observed differences. In addition, an ANOVA was conducted to compare the total number of nematodes (regardless species identity) in FiD with D and FiT with T. A Tukey Honest Significant Differences test was performed on the significant factors.

## Results

### Effect of temperature regime and interspecific interactions on the population performance of the species

Temperature regime had no effect on the juvenile or adult abundances of Pm I (Fig. 1a). However, the abundance of Pm I adults was influenced by interspecific interactions and time (Table 1). Lower abundances of adults and juveniles were found when all three species were present (treatment T) compared with the two other treatments (all $p < 0.03$). No significant interaction terms were found (Table 1).

Temperature regime also had no effect on juvenile and adult abundances of Pm IV, while interspecific interactions and time did affect adult and juvenile abundances (Table 1). For this species, however, the highest abundances of adults were found in the D treatment (Pm I and Pm IV together) ($p < 0.0001$) (Fig. 1b). Significant interaction terms were only found for the juvenile abundances between interspecific interaction treatment and time (Table 1). Pm IV juveniles had higher abundances in the D treatment (1121 ± 107.2 juveniles) compared with the M4 treatment (193 ± 69.6 juveniles) only after 7 days. In the T treatment, lower juvenile abundances (92 ± 55.4 juveniles) were present after 21 days compared with the M4 and D treatment (respectively 447 ± 83.1 and 1109 ± 191.7).

Temperature regime did not affect adult abundances of Pm III, but it did affect juvenile abundances dependent on time (Table 1, Fig. 1c). Numbers of Pm III adults were influenced by the interaction of time and interspecific interaction treatment , with more adults after 14 days when Pm III occurred alone (M3: 168 ± 17.7) compared to the treatment where Pm III was incubated together with the two other species (T: 47 ± 23.6) (pairwise PERMANOVA: $p=0.005$). This difference was not present after 7 or after 21 days. Time, temperature regime, interspecific interactions treatment, the interaction between time and temperature and the interaction between time and interspecific interactions treatment all had significant influences on the juvenile abundances of Pm III (Table 1). After 14 and 21 days, lower juvenile Pm III abundances were found in the T treatment (respectively 368 ± 160.9 and 287 ± 158.0) compared with the M3 treatment (respectively 1787 ± 354.9 and 1149 ± 176.9). After 14 days, more juveniles were found at the fluctuating temperature (1570 ± 457.5) compared with a constant temperature regime (584 ± 215.9). In tables 2 and 3, respectively, the average number of nematodes in the different treatments and an overview of the effect

of the interspecific interactions on the population performance of all three species can be found.

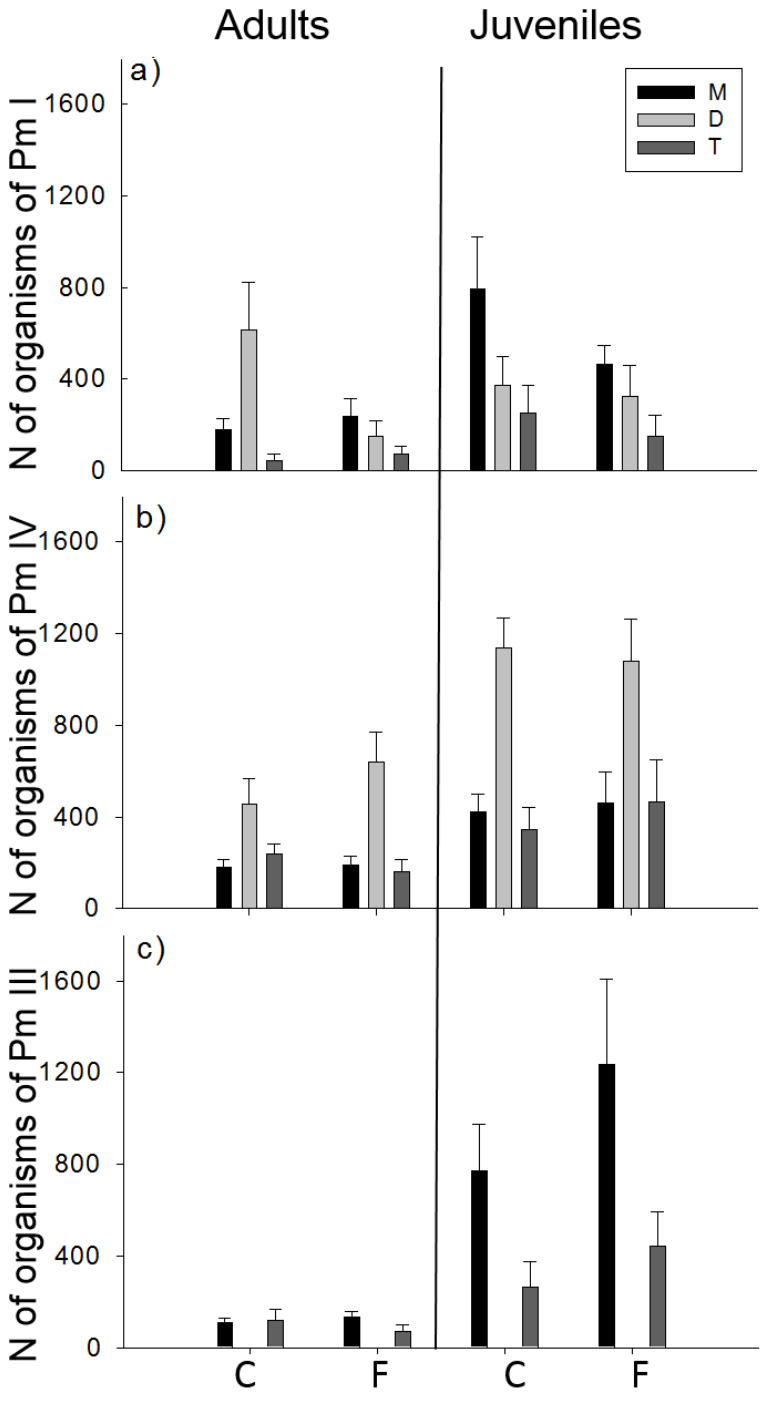



Figure 1: Time-averaged number of nematodes (± SE) of *Litoditis "marina"* species (adults and juveniles) in the different interspecific interaction treatments (three species: T, two species: D and one species: M) and different temperature treatments (F and C) for (a) Pm I, (b) Pm IV and (c) Pm III.

Table 1: Results of the within-species statistical analyses on population performance (independent factors: temperature (fluctuating vs. constant), interspecific interactions (M, D and T for Pm I and Pm IV; M and D for Pm III) and time; dependent factors: number of adults and juveniles in three cryptic species of *Litoditis "marina"*). Level of confidence = 95 %. Interspec.int.= interspecific interactions; temp.= temperature; p= statistical p value; F=F statistic.

| | | Pm I | | | | Pm IV | | | | Pm III | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Df** | | **Adults** | | **Juveniles** | | **Adults** | | **Juveniles** | | **Adults** | | **Juveniles** | |
| | Pm I / Pm IV (Pm III) | F | p | F | p | F | P | F | p | F | p | F | p |
| **Temp.** | 1 | 0.13 | 0.72 | 1.15 | 0.29 | 0.42 | 0.53 | <0.001 | 0.99 | 0.25 | 0.61 | 6.56 | **0.017** |
| **Intersp.int.** | 2 (1) | 19.71 | **<0.001** | 9.31 | **<0.001** | 15.93 | **<0.001** | 16.38 | **<0.001** | 1.22 | 0.23 | 27.61 | **<0.001** |
| **Time** | 2 | 6.10 | **0.005** | 2.95 | 0.07 | 5.42 | **0.009** | 5.33 | **0.009** | 3.11 | 0.07 | 14.90 | **<0.001** |
| **Temp.:Intersp.int.** | 2 (1) | 1.35 | 0.27 | 0.30 | 0.74 | 1.69 | 0.20 | 0.18 | 0.83 | 2.11 | 0.16 | 1.33 | 0.26 |
| **Temp.:Time** | 2 | 2.96 | 0.06 | 0.54 | 0.59 | 1.77 | 0.18 | 0.34 | 0.72 | 2.10 | 0.15 | 7.40 | **0.003** |
| **Intersp.int.:Time** | 4 (2) | 1.23 | 0.32 | 0.79 | 0.54 | 1.45 | 0.24 | 7.10 | **<0.001** | 4.40 | **0.02** | 16.90 | **<0.001** |
| **Temp.:Intersp.int.: Time** | 4 (2) | 2.05 | 0.11 | 0.65 | 0.63 | 0.41 | 0.80 | 0.13 | 0.97 | 2.98 | 0.07 | 2.72 | 0.09 |

Table 2: Number of nematodes ± SE (adults or juveniles) over time for the different temperature treatments (Co= constant; Fl= fluctuating) and interspecific interaction treatments.

| | Adults | M | | D | | T | |
|---|---|---|---|---|---|---|---|
| **Pm I** | | Co | Fl | Co | Fl | Co | Fl |
| | 7 days | 48 ± 14 | 89± 23 | 37± 18 | 74± 67 | 5± 4 | 55± 29 |
| | 14 days | 187 ± 94 | 300± 40 | 803± 347 | 78± 51 | 79± 40 | 1± 1 |
| | 21 days | 311± 25 | 293± 43 | 1003± 392 | 353± 142 | 83± 82 | 157± 86 |
| | Adults | M | | D | | T | |
| **Pm IV** | | Co | Fl | Co | Fl | Co | Fl |
| | 7 days | 89 ±44 | 62 ±32 | 428 ±32 | 350 ±54 | 317 ±60 | 142 ±97 |
| | 14 days | 257 ±37 | 300 ±40 | 590 ±254 | 994 ±197 | 228 ±90 | 316 ±38 |
| | 21 days | 201 ±11 | 205 ±48 | 344 ±263 | 570 ±259 | 168 ±85 | 26 ±15 |
| | Adults | M | | | | T | |
| **Pm III** | | Co | Fl | | | Co | Fl |
| | 7 days | 78±37 | 90±48 | | | 11±11 | 108±60 |
| | 14 days | 156±8 | 179±37 | | | 79±39 | 14±11 |
| | 21 days | 100±3 | 136±14 | | | 269±84 | 91±71 |
| | Juveniles | M | | D | | T | |
| **Pm I** | | Co | Fl | Co | Fl | Co | Fl |
| | 7 days | 99±45 | 286±46 | 118±21 | 67±21 | 92±54 | 52±18 |
| | 14 days | 986±463 | 818±288 | 537±330 | 606±280 | 150±93 | 292±287 |
| | 21 days | 1304±53 | 634±219 | 463±200 | 526±335 | 380±341 | 107±80 |
| | Juveniles | M | | D | | T | |
| **Pm IV** | | Co | Fl | Co | Fl | Co | Fl |
| | 7 days | 169±64 | 217±140 | 1030±124 | 1212±348 | 442±108 | 462±159 |
| | 14 days | 555±75 | 818±288 | 1220±403 | 978±334 | 454±246 | 886±451 |
| | 21 days | 537±149 | 356±63 | 1162±160 | 1056±394 | 140±114 | 44±10 |
| | Juveniles | M | | | | T | |
| **Pm III** | | Co | Fl | | | Co | Fl |
| | 7 days | 88±16 | 74±15 | | | 287±148 | 525±383 |
| | 14 days | 1018±189 | 2554±71 | | | 150±93 | 586±271 |
| | 21 days | 1213±288 | 1086±264 | | | 360±323 | 214±122 |

Table 3: Summary of the effect of interspecific interactions on population abundance (adults and juveniles) of the different cryptic species of *Litoditis "marina"* (Pm I, Pm III and Pm IV) in the D (Pm I + Pm IV) and T treatment (PmI, Pm III and Pm IV) compared with the M treatment (0= statistically no differences; -: lower abundance compared with M; +: higher abundance compared with M).

| | **Adults** | | **Juveniles** | |
|---|---|---|---|---|
| | **D** | **T** | **D** | **T** |
| **Pm I** | 0* | 0 | 0 | - |
| **Pm IV** | + | 0 | + | 0[b] |
| **Pm III** | NA | 0[a] | NA | -[c] |

[*] at constant temperature a positive effect for Pm I adults occurred

[a] at 14 days a negative effect occurred

[b] at 21 days a negative effect occurred

[c] at day 7 no difference was found

**Assemblage composition and dynamics**

In the treatment with two species, total abundances of adults and juveniles (regardless species) were affected by time and interspecific interactions treatment (see Table 4a), but not by temperature. Lowest abundances of nematodes occurred after 7 days. They did not differ between 14 and 21 days. Much higher numbers of nematodes were observed in the D treatment compared with the FiD treatment (respectively 930 ± 171.6 vs. 97 ± 83.9 adults and 1459 ± 95.1 vs. 1074 ± 274.6 juveniles (Fig. 2)). Comparing adult assemblage dynamics of Pm I and Pm IV between the D and FiD treatments showed significant effects of interspecific interactions, time and the interaction of interspecific interactions with temperature regime on the assemblage composition (Table 4b). Pm I became dominant over Pm IV in the D treatment at the end of the experiment at a constant temperature, but the opposite was true at a fluctuating temperature. In the fictitious treatment (FiD), however, Pm IV was not dominant over Pm I at this fluctuating temperature (Fig. 2a). For juvenile assemblage dynamics, only time and interspecific interactions were significant (Table 4b, Fig. 2b). A clear effect of interspecific interactions was shown, with Pm IV juveniles being dominant over Pm I in the D treatment compared with the FiD treatment independent of temperature regime.

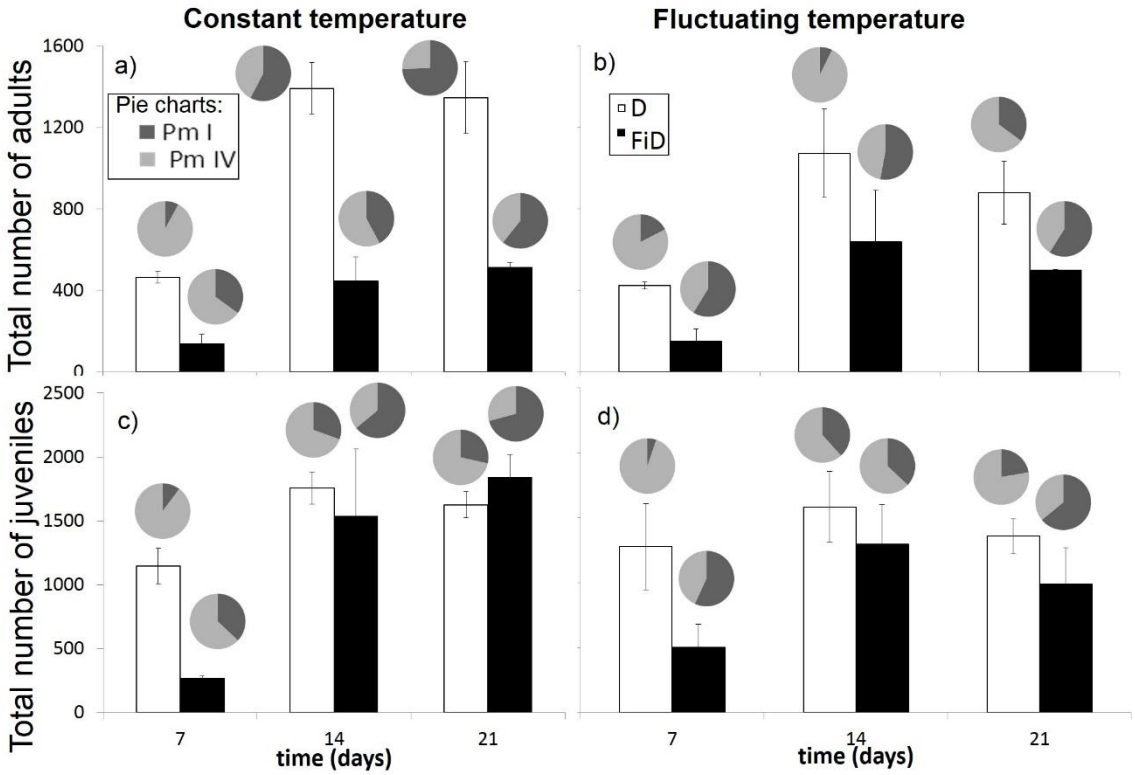



Figure 2: Total number of nematodes (adults: a + b, juveniles: c + d) over time (average ± SE) in the different temperature treatments (constant temperature: a + c, fluctuating temperature: b + d) with assemblage dynamics at the different sampling times for the D treatment (Pm I and Pm IV with interspecific interactions) and the FiD treatment (Pm I and Pm IV without interspecific interactions).

Table 4: Results of the statistics on A) total abundances regardless species (results of 3-way ANOVA's) and B) adult and juvenile assemblage compositions (3-way PERMANOVA). For both the effect of temperature (constant vs. fluctuating), interspecific interactions (fictitious vs. real populations) and time was studied for experiments with two species (D vs. FiD) and three species (T vs. FiT). Level of confidence = 95 %. Interspec.int. = interspecific interactions; temp.= temperature; p= statistical p value; F=F statistic.

| **A)** | | D compared with FiD | | | | T compared with FiT | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Total abundances** | Df | Adults | | Juveniles | | Adults | | Juveniles | |
| **regardless species** | | F | p | F | p | F | p | F | p |
| Temp. | 1 | 1.99 | 0.17 | 1.81 | 0.19 | 6.89 | 0.004 | 5.89 | **0.023** |
| Intersp.int. | 1 | 50.03 | **< 0.0001** | 7.10 | **0.013** | 0.16 | **0.69** | 17.35 | **< 0.0001** |
| Time | 2 | 24.30 | **< 0.0001** | 10.55 | **0.0005** | 4.60 | **0.004** | 17.71 | **< 0.0001** |
| Temp.:Intersp.int. | 1 | 5.12 | **0.03** | 0.40 | 0.53 | 5.05 | **0.034** | 0.41 | 0.53 |
| Temp.:Time | 2 | 0.84 | 0.44 | 2.20 | 0.13 | 2.36 | 0.12 | 8.46 | **0.002** |
| Interspec.int.:Time | 2 | 2.49 | 0.10 | 2.47 | 0.11 | 4.73 | **0.019** | 14.95 | **< 0.0001** |
| Temp.:Intersp.int.: Time | 2 | 0.91 | 0.42 | 0.51 | 0.61 | 2.17 | 0.14 | 1.19 | 0.32 |

| **B)** | | D compared with FiD | | | | T compared with FiT | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Assemblage** | Df | Adults | | Juveniles | | Adults | | Juveniles | |
| **composition** | | F | p | F | p | F | p | F | p |
| Temp. | 1 | 2.88 | 0.09 | 0.99 | 0.39 | 1.88 | 0.18 | 3.66 | **0.019** |
| Intersp.int. | 1 | 9.27 | **0.001** | 14.59 | **0.001** | 0.17 | 0.69 | 14.42 | **0.001** |
| Time | 2 | 5.38 | **0.002** | 3.88 | **0.01** | 6.88 | **0.004** | 9.01 | **0.001** |
| Temp.:Intersp.int. | 1 | 4.36 | **0.03** | 0.62 | 0.51 | 5.05 | **0.034** | 0.83 | 0.46 |
| Temp.:Time | 2 | 1.24 | 0.30 | 0.70 | 0.60 | 0.85 | 0.44 | 3.79 | **0.002** |
| Interspec.int.:Time | 2 | 1.01 | 0.40 | 1.26 | 0.28 | 4.73 | **0.019** | 7.69 | **0.001** |
| Temp.:Intersp.int.: Time | 2 | 1.22 | 0.33 | 0.76 | 0.49 | 2.17 | 0.14 | 1.34 | 0.24 |

In the treatments with three species, total numbers of adults (regardless species) were affected by time, the interaction between time and interspecific interactions treatment as well as the interaction between interspecific interactions treatment and temperature (Table 4a), with lower numbers of adults in the T treatment compared with the FiT treatment at fluctuating temperature at the end of the experiment (Fig. 3). In the T treatment, more adults were found at the constant temperature compared with the fluctuating temperature (respectively 358 ± 43.2 and 232 ± 43.0 adults). Total abundances of juveniles (regardless species) were affected by time, interspecific interactions treatment, temperature, the interaction between time and interspecific interactions treatment, and the interaction between time and temperature (Table 4a). Abundances of juveniles differed between the T and FiT treatment after 14 days and 21 days, with very low numbers of juveniles in the T treatment. Only at day 14, numbers of juveniles were higher in the fluctuating temperature regime compared with the constant temperature (Fig. 3). For the assemblage dynamics, no effect of temperature regime on the adult assemblages was found. There was an effect of time, interspecific interactions (T vs FiT) and the interaction of time and interspecific interactions (Table 4b) on the adult assemblages. Differences in assemblage dynamics were found between the FiT and the T treatment at every time moment (Fig. 3). SIMPER analysis showed that Pm I was the main responsible for the dissimilarity between these treatments. Pm I was less abundant in the T treatment than expected based on the FiT-treatments, in which Pm I became dominant after 14 days (Fig 3a + b), while Pm IV was the most abundant species in the T treatment after 7 and 14 days and Pm III after 21 days. Moreover, juvenile assemblage composition was also influenced by time, interspecific interactions, temperature, the interaction of time and interspecific interactions and the interaction of time and temperature (Table 4b, Fig. 3 c-d). The assemblage dynamics for juveniles differed between the FiT and T treatment and corresponded well with those observed in the adults. After 7 days, Pm I juveniles were less dominant in the T treatment than expected based on the FiT treatment, and after 14 days Pm IV was the most abundant species in the T treatment. After 21 days Pm III juveniles became more abundant compared with the other time moments (Pm III contributions to differences between time moments: all > 47.63%). The difference between the FiT and T treatment was mainly due to Pm IV (65.23% contribution to the dissimilarity). Temperature regime had an effect on day 14, when Pm III juveniles were more abundant at constant temperature compared with the fluctuating temperature (Pm III contributed 62.11 % to this dissimilarity).

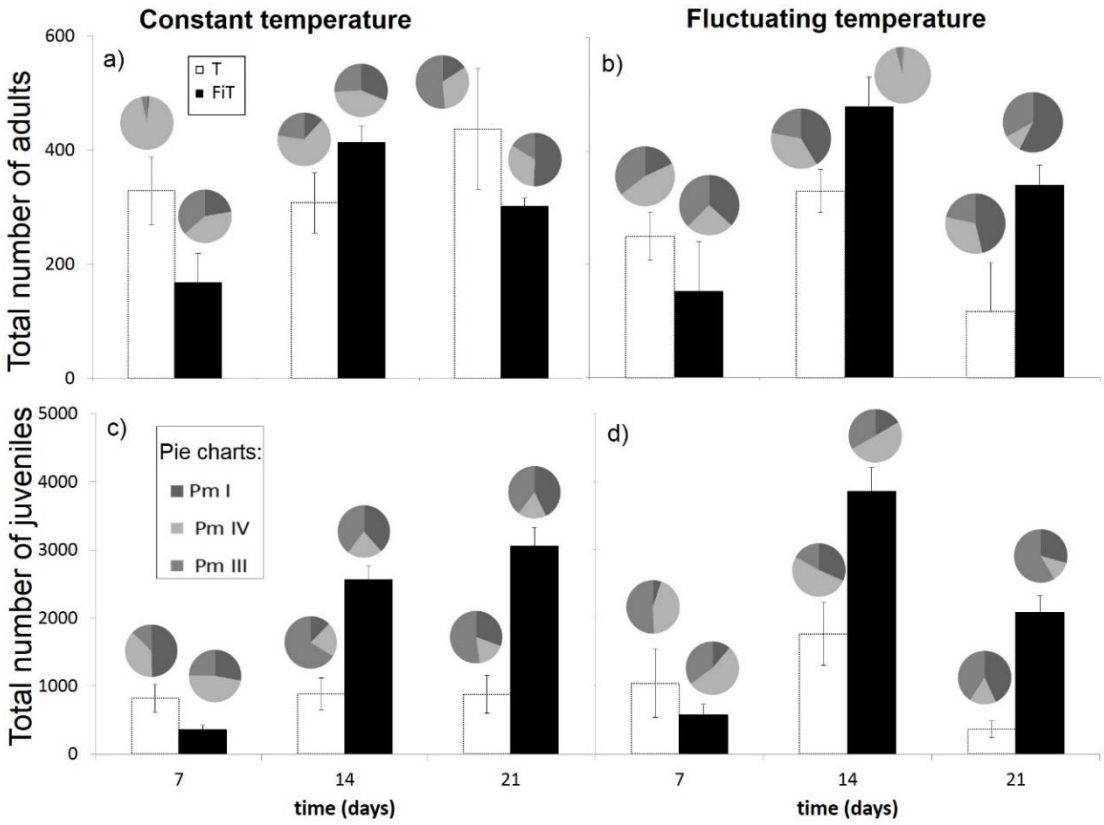



Figure 3: Total number of nematodes (adults: a + b, juveniles: c + d) over time (average ± SE) in the different temperature treatments (constant temperature: a + c, fluctuating temperature: b + d) with assemblage dynamics at the different sampling times: pie charts for the T treatment (Pm I, Pm IV and Pm III with interspecific interactions), and the FiT treatment (Pm I, Pm IV and Pm III without interspecific interactions).

## Discussion

The results of this study demonstrate that interspecific interactions rather than temperature regime governed assemblage dynamics of species mixtures. The effect of daily temperature fluctuations in the tested range on the population performance of cryptic species of *Litoditis "marina"* is limited and species-specific. However, assemblage dynamics of the species differed between fluctuating versus constant temperature, indicating that interactions between the species changed depending on the temperature regime.

### Fluctuating temperature and interspecific interactions affect population performance in a species-specific way

Fluctuating temperature had no differential effect compared with constant temperature on the population performance of Pm I and Pm IV populations. However, the population performance of Pm III was affected by temperature regime dependent on time: higher juvenile abundances occurred after 14 days under fluctuating temperature (Table 2). This could be the result of a positive effect of the maximum temperature on life-history traits, such as reproduction and development time (Atkinson, 1996). Experiments at constant temperatures have indeed shown that Pm III performs better at 25°C than at 15°C (De Meester, et al., 2015a; chapter III), and that this effect is more pronounced than in the other cryptic species. Moreover, phylogeographic data show the presence of Pm III in regions with higher average temperatures, where the other species were absent (Derycke, et al., 2008b). This may indicate that Pm III is better adapted to higher temperatures than the other cryptic species, while it does not perform worse than the other species at lower temperatures (15°C and 20°C (De Meester, et al., 2015a; chapter III)). However, the higher abundance of Pm III juveniles at fluctuating temperature was a transient feature only found after 14 days, which could also point to a stress response (different conditions compared with the stock culture). Hence, temperature regime had only limited effects on population performance of any of the three *Litoditis* species in monoculture.

In contrast, interspecific interactions clearly influenced the population performance of all three species, both positively and negatively. Decreased population sizes are the result of interspecific competition between the species, which can be due to reductions in survival, growth or fecundity (Begon, et al., 2009). Competition was asymmetrical, mainly affecting the abundances of Pm I and Pm III juveniles but not those of Pm IV. Asymmetrical competition has also been found among other bacterial-feeding free-living nematodes (Postma-Blaauw, et al., 2005; dos Santos, et al., 2009). In contrast, Pm IV appeared to benefit

from the presence of Pm I (D treatment), suggesting some sort of facilitative interaction (Bruno, et al., 2003). Over time, the interspecific interaction effect sometimes changed (Table 3), which indicates that population dynamics are still changing and that longer-term studies can be important to properly predict the outcome of those interactions.

**Fluctuating temperature alters some interspecific interactions**

Temperature fluctuations altered interactions between Pm I and Pm IV in the two-species treatment but not in the three-species treatment. Pm I and Pm IV are phylogenetically more closely related to each other than to other cryptic *Litoditis "marina"* species, and we therefore expected stronger competition between them according to the competition-relatedness relationship (Darwin, 1859). Indeed, in a previous competition experiment with four cryptic species in closed microcosms under constant environmental conditions, Pm IV was completely outcompeted (De Meester, et al., 2011; chapter II) and Pm I was still very abundant. The current experiment contradicts our prediction: Pm I and Pm IV were able to coexist in high abundances, even under constant temperatures, suggesting that Pm I was not the main competitor of Pm IV in our earlier experiment, and/or that the presence of additional species changes the type of their interaction. In fact, at a constant temperature, both Pm I and Pm IV attained higher population abundances when they occurred together (without the third species, D treatment) (for adults and juveniles in Pm IV, only for adults in Pm I), suggesting a sort of facilitative mutualism (Bruno, et al., 2003; Valiente-Banuet & Verdú, 2008). Higher total nematode densities at the start of the experiment can potentially affect bacterial growth and abundance through grazing or mucus production (Moens, et al., 2005) and could thus have increased food availability and enhanced nematode growth in both species. At fluctuating temperature, the facilitative effect was still pronounced for Pm IV, but disappeared for Pm I. Pm IV now became more abundant than Pm I, pointing at a facilitative commensalism, with a positive effect of Pm I on Pm IV, and no effect of the presence of Pm IV on Pm I. Temperature regime thus altered the interaction between these two species from a sort of mutualism to commensalism, demonstrating that interactions between the species can change depending on the abiotic environment (Dunson & Travis, 1991). Such environmental impacts on species interactions could result from species-specific responses to the abiotic environment. However, in this experiment, no significant differences in population performance were found in the monospecific treatments at fluctuating temperature compared with the constant temperature. Experiments on their life history at constant temperatures (15°C, i.e. the lowest temperature in our F treatment, 20°C

and 25°C, i.e. the highest temperature in our F treatment) revealed no obvious differences in juvenile development time, reproduction rate or total population development between these two *L. "marina"* species (Pm I and Pm IV) (De Meester, et al., 2015a; chapter III), suggesting that differences in their life histories at these temperatures are negligible. Nevertheless, some studies on fish and butterflies have shown that fluctuating compared to constant temperatures caused shorter development times (Fischer, et al., 2011), and we did not include development or generation time as life-history traits in our present experiment. Hence, further investigation on the effect of fluctuating temperature on generation time is needed to check if the difference in interactions is the result of differences in life history. Another possibility is a direct effect on the interspecific behaviour of the two species with the abiotic factors having an effect on the way species interact with each other (Dunson & Travis, 1991), for instance by influencing interference behaviour (Amarasekare, 2002). Additionally, the result of the interspecific interactions was not fully consistent among adults and juveniles: whereas adult abundances of both Pm I and Pm IV were higher in the combination treatment (D, a sort of mutualism), only Pm IV juveniles were more abundant in the D than in the M treatment (facilitative commensalism). Processes as maturation, reproduction and mortality could be differentially influenced at each stage of the individual by interspecific competitive interactions (Mougi & Nishimura, 2005). Valiente-Banuet and Verdú (2008) demonstrated in plants that interactions can alter along their development and/or in response to temporal fluctuations of the environment. In this experiment, an effect of both could be found: differential interactions between adults and juveniles of the different species were found under certain abiotic conditions. Juveniles have often been demonstrated to be more sensitive to various kinds of environmental stress than adults (Martinez, et al., 2012), which may contribute to such differential interactions.

The interactions between the species changed when three species were present (T treatment), and temperature did not alter these interactions; however, the total number of nematodes (regardless species) was affected by the temperature regime. Over time, the dominance of the species changed in adult and juvenile assemblages in both temperature regimes: in the beginning, Pm IV was the most abundant species, whereas after 21 days Pm III became the most abundant one. This suggests that the community was still changing after three weeks. For Pm III juveniles this dominance occurred faster for the constant temperature (already at 14 days) compared with the fluctuating temperature. However, Pm I and Pm III showed lower abundances in the T treatment compared to the respective monospecific treatments

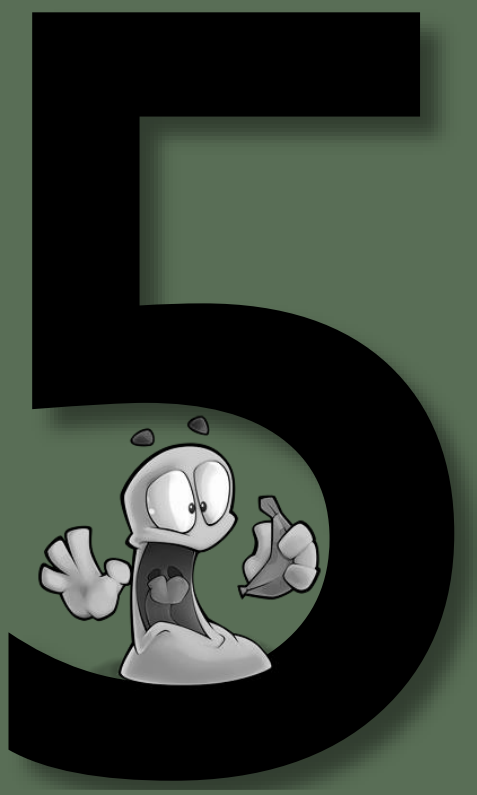
(M1 and M3) (Fig. 1), which can be the result of competition between these two species. No effect of Pm I and Pm III on Pm IV was found. These results are in conflict with a previous study (De Meester, et al., 2011; chapter II) in which the same species, together with a fourth one (Pm II), were simultaneously inoculated into closed microcosms. In that experiment, Pm II and Pm IV adults went extinct after 35 days at a constant temperature of 20°C, whereas no substantial effect of competition on Pm I and Pm III was evident. Perhaps the time frame of the present experiment was too short for this competitive effect to become manifest. The fact that Pm IV juvenile abundance dropped after 21 days in the T treatment compared to the M4 treatment may point to this explanation. Alternatively, the extinction of Pm IV in that previous experiment could have mainly resulted from competition with Pm II and/or Pm II could have changed the interactions between the other species. It seems that there are complex interactions between the species, which are not just the sum of their separate pairwise interactions. As a result, a competitively intransitive network, in which species' abilities cannot be ranked in a hierarchy (Rojas-Echenique & Allesina, 2011), exists in this cryptic species complex. An addition of one species to a community can change all existing interactions between the others. One species can alter the effect that another species has on a third one, and thus pairwise species interactions are influenced by the presence and density of other species in the community. These indirect effects may importantly affect the success of a species (Higashi & Patten, 1989). Moreover, total number of juveniles was lower when interspecific interactions occurred, again pointing to the fact that juveniles may be more sensitive to stress (Martinez, et al., 2012). Altough there was no clear effect of temperature regime on the assemblage dynamics, fluctuating temperature had an effect on the total abundances of nematodes over time (regardless species) in the T treatment, with a decrease in abundances by the end of the experiment compared with the constant temperature. This could point out that the competitive interactions will be more severe for all species at fluctuating temperature, without affecting the relative contribution of each species. Salinity already proved to have an effect on the strength of the interactions between *Litoditis* species (De Meester, et al., 2011; chapter II), showing that differences in abiotic parameters can change the strength of interspecific interactions.

**Conclusions**

The results of this experiment indicate that fluctuating temperature only had a small effect on the population performance of one of the three cryptic species studied here, but did influence the assemblage composition of the species. Depending on the species combination, interspecific interactions changed or became more severe when temperature fluctuations occurred. This indicates that there is a complex interaction between abiotic (temperature) and biotic (interspecific interactions) factors. As a consequence, the outcome of interactions cannot be easily predicted. and a competitively intransitive network between the cryptic species of *Litoditis "marina"* may exist. In natural situations fluctuations in the biotic and abiotic regulators can alter the outcome of the interactions between species and may facilitate coexistence.

## Acknowledgements

Funding for this research was obtained from the Flemish Fund for Scientific Research (FWO) through project 3G040407, and from Ghent University through projects 011060002 and 01GA1911 W. The authors would like to thank Wouter t'Jonck for his help in qPCR analyses.



# CHAPTER V

# DIFFERENTIAL RESOURCE UTILIZATION AMONG CRYPTIC SPECIES

---

Slightly modified from:

Derycke, S., De Meester, N., Rigaux, A., Creer, S., Bik, H., Thomas, W.K. & Moens, T. (2016) Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability. *Molecular Ecology*: doi: 10.1111/mec.13597

*Nele De Meester performed the food experiment and analysed the MiSeq data. She also critically revised the manuscript, and contributed to the final version of the manuscript.*

## Abstract

Differences in resource use or in tolerances to abiotic conditions are often invoked as potential mechanisms underlying the sympatric distribution of cryptic species. Additionally, the microbiome can provide physiological adaptations of the host to environmental conditions. We determined the intra- and interspecific variability of the microbiomes of three cryptic nematode species of the *Litoditis "marina"* species complex that co-occur, but show differences in abiotic tolerances. Roche 454 pyrosequencing of the microbial 16S rRNA gene revealed distinct bacterial communities characterized by a substantial diversity (85 – 513 OTUs) and many rare OTUs. The core microbiome of each species contained only very few OTUs (2 – 6), and four OTUs were identified as potentially generating tolerance to abiotic conditions. A controlled experiment in which nematodes from two cryptic species (Pm I and Pm III) were fed with either an *E. coli* suspension or a bacterial mix was performed and the 16S rRNA gene was sequenced using the MiSeq technology. OTU richness was 10 fold higher compared to the 454 dataset and ranged between 1118 – 7864. This experiment confirmed the existence of species-specific microbiomes, a core microbiome with few OTUs, and high interindividual variability. The offered food source affected the bacterial community and illustrated different feeding behaviour between the cryptic species, with Pm III exhibiting a higher degree of selective feeding than Pm I. Morphologically similar species belonging to the same feeding guild (bacterivores) can thus have substantial differences in their associated microbiomes and feeding strategy, which in turn may have important ramifications for biodiversity – ecosystem functioning relationships.

## Introduction

Many taxa contain species that are morphologically (nearly) identical but show genetic differences in neutral markers that are comparable to, or greater than, those observed between species with distinct morphologies. These cryptic species have been observed in all major taxa and in all biogeographic regions (Pfenninger & Schwenk, 2007). Despite their morphological similarity, cryptic species can have distinct evolutionary histories of millions of years (Elmer, et al., 2013; Glasby, et al., 2013; Perez-Portela, et al., 2013). The conservation of the morphological pattern results from selection-promoting morphological stasis and/or from a differentiation in other characters that are invisible to the human eye (Bickford, et al., 2007). In the marine environment, cryptic species of benthic invertebrates often show a sympatric distribution, but at the same time pronounced habitat preferences defined by depth, salinity, temperature and substrate (Knowlton, 1993). Next to these abiotic parameters, intrinsic differences between cryptic species, such as the differential use of resources or the presence of distinct microbiomes (both in the gut and on the cuticle), may impact the sympatric distribution of cryptic species as microbiomes can affect the physiology of the host (Cabreiro & Gems, 2013; Sison-Mangus, et al., 2014) which may have cascading effects on ecological interactions.

Substantial cryptic diversity has been observed in the phylum Nematoda (Sudhaus & Kiontke, 2007; de Leon & Nadler, 2010; Derycke, et al., 2013; Ristau, et al., 2013). In marine sediments, nematodes abound both in numbers and in local species diversity, with several tens of species co-occurring at submeter scales (Heip, et al., 1985). Nematode community composition, assessed through morphological characters, can be linked to physico-chemical characteristics of the sediment (Steyaert, et al., 1999; Vanaverbeke, et al., 2000), and at very small spatial scales, microhabitat differences can substantially alter nematode communities (Fonseca, et al., 2010; Gingold, et al., 2011). Based on the shape of the buccal cavity and the presence/absence of armature in the stoma, marine nematodes have been divided into feeding guilds (Wieser, 1953; Moens & Vincx, 1997). Nematodes without buccal armature can feed on bacteria and protists, while those having buccal armature can feed on microalgae (e.g. diatoms), on micro-invertebrates including nematodes and on other resources (Moens & Vincx, 1997). The niches of nematode species delineated by morphology are thus determined by a series of abiotic and biotic parameters, but the extent of niche breadth of, and niche differences between sympatrically occurring cryptic nematode species remain unknown. Moreover, the nematode microbiome influences the physiology of the worm and

impacts its longevity (Cabreiro & Gems, 2013) and may, especially in the case of bacterivorous nematodes, be linked to the diet of the nematodes. Techniques currently available to assess resource use in minute organisms (e.g. stable isotope analysis) are unable to distinguish individual resource (Carman & Fry, 2002). The advances in high throughput sequencing now allow to more deeply investigate the microbial communities associated with sympatric bacterivorous nematode species to determine the extent of resource differentiation (bacteria related to food) and of microbiome differentiation (the microbiome 'sensu lato', which comprises the bacteria related to food and the microbiome 'sensu stricto' containing the commensal bacteria).

The bacterivorous marine nematode *Litoditis "marina"* (Bastian, 1865) Sudhaus, 2011 consists of at least 10 cryptic species (Derycke, et al., 2008b), three of which (Pm I, Pm II and Pm III) frequently co-occur on seaweed stands and deposits in the coastal area of Belgium and The Netherlands (Derycke, et al., 2005). In this region, the most abundant seaweeds typically belong to the genus *Fucus*. Phylogenetic analyses of mitochondrial and nuclear genes have revealed that Pm III is more distantly related to Pm I and Pm II (Derycke, et al., 2008b). Morphological differentiation between the three species is limited and requires a combination of morphometric characters (Derycke, et al., 2008a). No cross breeding between the species has been observed under laboratory conditions (Derycke, et al., 2008a; Derycke, unpublished data). Their coexistence implies that local populations of the three sympatric species experience (nearly) identical sets of abiotic factors like salinity and temperature. Nevertheless, both factors differentially impact demographic traits of the three species, resulting in a significantly lower minimum juvenile development time at higher temperatures and the production of more offspring at lower salinities for Pm III (De Meester, et al., 2015a; chapter III). Whether these species have a microbiome and whether such a microbiome would differ between species remains unknown. Furthermore, competitive interactions have been observed between these cryptic species (De Meester, et al., 2011; chapter II) and the presence of a bacterial food source impacted their dispersal behaviour (De Meester, et al., 2012; chapter VI). In addition to abiotic factors, niche differentiation between the cryptic species may thus be linked to resource divergence. Chemotaxis and tracer experiments with the cryptic *L. "marina"* species and other bacterivorous nematodes have shown that they can selectively migrate towards and/or feed on bacterial strains (Derycke, personal observations; Moens, et al., 1999; Estifanos, et al., 2013). If such selective feeding is present in sympatrically distributed cryptic nematode species, this would

support the idea that niche partitioning is an important process allowing their coexistence. Bacteria are the main food source of *Litoditis "marina"*, but occasionally also small green algae are taken up (Moens & Vincx, 1997). As such, *L. "marina"* is considered to be a deposit feeder (Moens & Vincx, 1997). The oesophagus contains a distinct middle bulb and a poorly developed posterior bulb with valves (Inglis & Coles, 1961) which is very similar to the oesophagus of *C. elegans* and which grinds the bacteria before transmission to the intestine (Seymour, et al., 1983). The microbiome 'sensu lato' may thus also be linked to feeding behaviour.

The aim of this study was to characterize the bacterial communities associated with co-occurring cryptic nematode species to reveal the extent of intra- and interspecific differentiation in the microbiome under natural field conditions. Single nematode specimens from each of three co-occurring species were simultaneously isolated from the same habitat in the same location, and a fragment of the microbial 16S rRNA gene was sequenced using the 454 GS FLX system (Roche). Next, to test whether the observed differences in bacterial communities are linked to resource use, we conducted a laboratory experiment with Pm I and Pm III nematodes which had been starved for two days before offering those *Escherichia coli* or a diverse bacterial mix. We expected to find significant differences in OTU composition between the two food treatments if the bacterial communities detected with the NGS approach indeed reflect resource use. Moreover, selective feeding can be detected if different species use different OTUs out of the offered food sources. In addition; when significant differences between species irrespective of food are found, this may indicate the presence of species-specific microbiomes, which may help explain their differences in abiotic tolerances (Cabreiro & Gems, 2013).

# Material and methods

## Specimen collection

Individual nematode specimens have been collected in the framework of a geographical and seasonal investigation of the population genetic diversity in coastal and estuarine environments in Belgium and the southwest of The Netherlands in 2003 (Derycke, et al., 2006). This study revealed that three closely related, cryptic *Litoditis* species (at that time *Pellioditis* marina) were co-occurring in the Paulina saltmarsh (51°21'N, 3°49'E) in October 2003 (Appendix S1). Fragments of living *Fucus* sp., one of the preferred habitats for *L. "marina"*, were randomly collected and incubated on agar slants (Moens & Vincx, 1998). Nematodes were subsequently allowed to colonize the agar for about two days, during which they were able to feed on the natural bacteria associated with the *Fucus* fragments. No *E. coli* was added to these agar slants. After two days, specimens belonging to the *L. "marina"* species complex were identified under a dissecting microscope using diagnostic morphological characters (Inglis & Coles, 1961) and handpicked from the agar with a fine needle. All worms were digitally photographed using light microscopy, and stored individually in 70 – 95 % acetone until processed. Specimens were then assigned to cryptic species based on the COI genotyping from the population genetic survey (Derycke, et al., 2006). We randomly selected six nematode specimens each of Pm I, Pm II and Pm III from the Paulina marsh samples.

### *A. DNA extraction and nematode identification*

DNA was extracted using a simple lysis procedure by transferring individual nematodes to Worm Lysis Buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % NP40, 0.45 % Tween20). The worms were then cut in pieces with a razor blade, frozen for 10 min at -20 °C and subjected to proteinase K (60 µg/ml) treatment. Finally, the DNA samples were centrifuged for 1 min at maximum speed (13200 rpm) and the supernatant was used in the subsequent PCR. In the original study, the mitochondrial cytochrome oxidase c subunit 1 (COI) gene was amplified and analysed using Single Strand Conformation Polymorphism (Derycke, et al., 2006). To double-check species identity, we re-amplified and sequenced the COI gene of all specimens for which we still had sufficient DNA. PCR amplification was done in 25 µl PCR reactions for 35 cycles, each consisting of a 30 s denaturation at 94 °C, 30 s annealing at 50 °C, and 30 s extension at 72 °C, with an initial denaturation step of 5 min at 94 °C and a final extension step of 5 min at 72 °C. Primers JB3 and JB5 were used (Derycke, et al., 2006) and unidirectional Sanger sequencing was done with JB3 by

Macrogen. The obtained sequences were then compared to published sequences of the *Litoditis "marina"* species complex (Derycke, et al., 2008a). All samples used in this study had COI sequences that matched the SSCP based identification.

### *B. 16S rRNA gene amplification and 454 GS FLX sequencing of individual nematode specimens from the field*

The bacterial communities associated with the six specimens from each of the three co-occurring nematode species Pm I, Pm II and Pm III were characterized through amplification of a portion of the 16S rRNA gene using the DNA extracts from the previous study. The 16S rRNA gene was amplified using primers 968F and 1401R (Zoetendal, et al., 1998). Amplification was done in 50µl reactions containing 37.3 µl water, 5 µl buffer (10X), 1 µl dNTPs (10mM each), 2 µl of each primer (10 µM) 0.2 µl Toptaq polymerase (Qiagen) and 2.5 µl DNA. Cycling conditions consisted of an initial denaturation of 2 min at 95°C, followed by 35 cycles of 95°C for 1 min, 53°C for 45s, 72°C for 3 min, and a final extension of 72°C for 10 min. The number of cycles follows that of other environmental bacterial surveys (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). The forward primer contained the Roche A adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and an 11 bp MID tag, while the reverse primer contained the Roche B adaptor (CTATGCGCCTTGCCAGCCCGCTCAG) and an 11 bp MID tag. The MID tags are provided in appendix S2 and allowed separation of the sequences according to the nine nematode specimens. The resulting fragment was 505 bp long. A 'no template' control was included for each primer set to ensure no contamination occurred in the lab. PCR products were checked on 1% agarose gels, purified with AMPure beads following the manufacturer's protocol (Beckman Coulter Inc.), and measured with a Qubit fluorometer (Life technologies). Samples were then pooled in equimolar concentrations and loaded on the Bioanalyzer (Agilent Technologies) to check the presence of a single peak. The pooled sample was bidirectionally sequenced on 1/8 of a 454 GS FLX plate (Macrogen). Two runs were performed, each containing three specimens from each species.

**Data analysis**

The raw datasets from the two runs were filtered and denoised with FlowClus (Gaspar & Thomas, 2015), a program that uses the flow information in the sff.file to screen and correct errors. FlowClus is available for downloading at http://sourceforge.net/projects/flowclus/. Primers and barcodes were removed from the sequences and the reverse complement was taken of the reverse sequences. Filtering involved removal of sequences that were outside the 200 - 1000 bp range, had an average quality less than 25, or contained more than six homopolymers. Denoising was chosen with a constant value of 0.5. Chimera's were detected using Uchime without reference database (Edgar, et al., 2011) and removed from the dataset. The sequences were then processed using QIIME 1.9.0 (Caporaso, et al., 2012). Forward sequences from both runs were merged to create a dataset with only forward sequences. The reverse sequences from both runs were also merged to create a dataset with only reverse sequences. Unlike for the paired-end reads generated with Illumina, the forward and reverse datasets generated by the 454 protocol cannot be merged because forward and reverse reads are not generated from the same PCR molecule. Therefore, the resulting forward and reverse datasets were independently clustered into OTUs with 97% similarity using an open-reference OTU picking strategy. OTUs that were only observed once in the total dataset were removed because these are most likely to represent sequencing errors or rare variants within genomes. Default settings of QIIME 1.9.0 were used, except for the subsampling in the open reference OTU picking strategy, which was set at 0.01 instead of 0.001. The number of sequences and OTUs obtained for each of the 18 specimens is summarized in appendix S3 in Supporting Information.

Taxonomy was assigned up to species level using the assign_taxonomy.py script and the 97% taxonomy and OTU files of the Greengenes 13.8 database, using the default settings of the Uclust algorithm as implemented in QIIME. When no hit was observed, OTUs were labelled as 'Unassigned'. The taxonomic compositions associated with each of the three nematode species were visualized through bar graphs in excel using the unrarefied dataset for both F and R datasets.

Diversity within and between the three cryptic species was compared. To account for differences in number of sequences for each specimen, the dataset was rarefied at 600 sequences per specimen for each dataset. This number was slightly lower than the lowest number observed in our samples (626 for the Forward dataset, 643 for the Reverse dataset, see appendix S1). Alpha diversity (Shannon Wiener, observed OTUs, Good's coverage) was

calculated using alpha_rarefaction.py in QIIME. Rank abundance graphs were constructed to explore the abundance of OTUs associated with each nematode specimen. Generalized UniFrac distances ($\alpha = 0.05$) (Chen, et al., 2012) were calculated with the GUnifrac package in R (R Development Core Team, 2008). Permanova was conducted on these UniFrac distances with species as grouping variable using the Adonis package in R. Permdisp and pairwise difference tests were also performed in R. Principal coordinates analysis (PCoA) plots were generated to visualize intra- and interspecific differences between the treatments using the Ade4 package in R. In addition, we investigated whether differences between species were caused by differences in rare OTUs, by constructing a dataset with only those OTUs that had at least 108 sequences (i.e. 1% of the rarefied dataset, which contained 18*600= 10800 sequences). This resulted in a forward and reverse dataset containing 18 OTUs with a frequency higher than 1%. Statistical analyses on these datasets were performed as described above.

To investigate whether each of the nematode species had bacterial OTUs that were present in all specimens of that particular species (= the core microbiome of each species), we ran the compute_core_microbiome.py script. The frequency of the core OTUs in each specimen was visualized using the sequence counts from the rarefied biom table. Because many bacterial strains show a lower than 3% divergence, we investigated whether the core community would be impacted by clustering OTUs at 99% instead of 97%. For this, we reran the open-reference OTU picking strategy for the reverse dataset using a similarity of 99%. Taxonomic assignment was done using the 99% taxonomy and OTU files of the Greengenes 13.8 database. All other settings and parameters and core microbiome analysis were identical as mentioned above.

Biomarker taxa that are most likely to explain differences in microbiome between the three nematode species were assessed using the Linear discriminant analysis Effect Size (Segata, et al., 2011) module as implemented in Galaxy (https://huttenhower.sph.harvard.edu/galaxy). Default settings were used, and species were selected as Class and specimens as subjects. We used the rarefied reverse dataset clustered at 97%.

**Food experiment**

To investigate whether the bacterial communities associated with the nematodes were part of the diet, living worms of Pm I and Pm III were subjected to two different food treatments: an *E.coli* treatment (Pm1E and Pm3E) and a 'bacterial mixture' treatment (Pm1B and Pm3B) in which nematodes were fed a natural inoculum of bacteria from the field. Fragments of the seaweed *Fucus* sp. from Paulina were put in culture flasks with artificial seawater (ASW) with a salinity of 25 for one week at a temperature of 15°C and afterwards rinsed in ASW with a salinity of 25. The ASW from the culture flasks and the washing step was filtered three times over a GF/C filter with a diameter of 1.2 µm to remove organisms with sizes exceeding those of bacteria, and frozen at -20°C until the experiment started. Two times 5 µL of this suspension was used for DNA extraction for later bacterial diversity analysis ('bacterial mixture'). Four Petri dishes of 5 cm inner diameter were filled with 4 mL of 1% bacto agar medium (salinity of 25 and buffered at a pH of 7.5 – 8 with TRIS-HCl in a final concentration of 5mM). Two dishes received 50 µL of a suspension of frozen-and-thawed *E. coli* (strain K12 in PBS buffer) with a density of $3x10^9$ cells $ml^{-1}$ to which either 20 adult Pm I or 20 adult Pm III nematodes were added. The two remaining dishes received 50 µL of the bacterial mix prepared from the *Fucus* thalli to which either 20 adult Pm I or Pm III nematodes were added. Monospecific cultures of the two cryptic species were raised from one single gravid female per species collected from Paulina marsh (The Netherlands) in March 2014 and maintained on sloppy (0.8%) nutrient:bacto agar media (temperature of 20°C; salinity of 25) with unidentified bacteria from their habitat as food (Moens & Vincx, 1998). Two pieces of agar of each nematode culture (Pm I and Pm III) were subjected to a DNA extraction and 16S rRNA gene amplification to pinpoint the bacteria that are able to grow on the culture medium. Nematodes were allowed to feed on the bacteria for two days, after which ten nematodes per treatment were picked out and quickly washed in cold sterile ASW to remove most of the adherent bacteria. Subsequently, they were put individually in 20µL WLB for DNA extraction. The DNA extraction was the same as described for the field specimens. For the pure bacterial mixture a DNA clean-up (Wizard) was necessary after the DNA extraction, due to the high salt concentration in the solution. In total, 46 DNA extracts were prepared (10 for each of the four food treatments, 2 from the agar from each stock culture, and 2 from the bacterial mixture).

### A. *16S rRNA amplification and Illumina MiSeq sequencing of individual nematode specimens from the food experiment*

For the DNA amplification and Illumina MiSeq sequencing a slightly adapted version of the protocol of the Earth Microbiome Project (Gilbert, et al., 2014) was used. Amplification was done in 20µl reactions containing 11.4 µl water, 4µl 10X buffer, 0.4µl dNTP's (10 mM), 0.2µl Phusion (high fidelity) polymerase, 2µl DNA template and l µL forward and 1µl reverse primer (both 10µM). The forward primer contained the 5' Illumina adaptor, forward primer pad and linker and the 515f primer. The reverse primer consisted of the reverse complement of the 3' Illumina adapter, the reverse primer pad and linker, the 806r primer and a Golay barcode. This Golay barcode was unique for each sample and the first 52 barcodes of the Earth Microbiome Project were used (Caporaso, et al., 2012). Cycling conditions consisted of an initial denaturation of 30s at 98°C, followed by 35 cycles of 98°C for 10s, 65°C for 30s, 72°C for15s, and a final extension of 72°C for 10 min. Samples were amplified in triplicates. Three samples were randomly chosen in which the triplicates received different barcodes to allow investigation of PCR cycle bias. We did detect some PCR bias, but most OTUs were shared between replicas and OTUs uniquely found in one replica reached only very low frequencies (maximum of 0.21%). All analyses regarding the technical replicates can be found in appendix S4. After amplification, triplicates were combined. PCR products were cleaned by selecting the correctly sized bands (300 – 350 bp) with the help of Clone-Well Agarose Gels (E-Gel). After this, the PCR concentration was measured with the Qubit Fluorometer (Life Technologies) and an equal amount of amplicon from each sample was pooled into one single, sterile tube. The final sample was checked for concentration and quality with the BioAnalyzer (Agilent Technologies). Illumina MiSeq sequencing was performed by the Genomics Core (UZ Leuven). Because only a small amount of reads from the nematodes fed *E. coli* were assigned to Enterobacteraceae (see results), the *E. coli* suspension was sequenced in a separate MiSeq run (as part of a follow-up experiment) to exclude any methodological issues. Three biological replicas of the suspension were amplified and sequenced as described above.

*B. Data analysis*

The Illumina paired-end sequences were first assembled with PEAR (Paired-end reader merger (Zhang, et al., 2014)). Subsequent filtering involved trimming of reads with a quality score of 25, read lengths had to be in the 200 -1000 bp range, and all reads containing uncalled bases were discarded. Subsequently, forward and reverse primers were removed with Cutadapt (Martin, 2011). The sequences were then processed using QIIME 1.8.0 (Caporaso, et al., 2010) with an open reference OTU picking strategy (97% clustering) as described above. Beta diversity analyses involved rarefaction of the dataset at 41000 sequences for each sample. Generalized UniFrac distances ($\alpha = 0.05$) (Chen, et al., 2012) and statistical analyses were calculated in R as described above. The technical replicates that received a different barcode to investigate PCR bias were merged into a single sample for alpha and beta diversity analyses. The rarefied dataset was also used to identify biomarker taxa between Pm I and Pm III related to resource use using species as class, food treatment as subclass and specimens as subjects. Default settings were used.

The *E. coli* samples were separately analysed from the first MiSeq run, but the same assemblage, filtering, trimming, OTU clustering and taxonomic assignment procedures were used.

**Scanning Electron Microscopy (SEM)**

In our previous study, all specimens were photographed digitally prior to the DNA extraction to have a morphological reference before being stored in acetone. To assess the abundance of bacteria associated with the nematode cuticle, we re-examined the digital pictures of the specimens used for next generation sequencing. In addition, nematodes grown on agar media with unidentified bacteria from their habitat and *E. coli* as additional food, from monospecific cultures of each of the three nematode species were used to generate SEM pictures of the head, tail and midbody region. These SEM pictures were generated to investigate the abundance and diversity of bacteria on the cuticle of the nematodes. The numbers of females photographed were 7, 3 and 7 for Pm I, Pm II and Pm III respectively, and the numbers of males were 9, 4 and 3, respectively. SEM pictures were generated with the JEOL JSM-840 scanning electron microscope by the Nematology Unit of the Biology Department at Ghent University.

# Results

## 16S rRNA composition of individual nematode specimens from the field

### *A. Taxonomic composition of the bacterial communities associated with cryptic species*

Taxonomic assignments at the phylum level were highly comparable for the Forward and Reverse datasets and only differed in the presence of an additional three 'phyla' ('unidentified bacteria', Planctomycetes and 'TM6') in very low frequency in the Reverse dataset. We restrict the detailed description of the taxonomic composition to the Reverse dataset, because it yielded slightly more sequences for each sample (Appendix S3). Taxonomic composition at the phylum level for the forward dataset can be found in appendix S5.

The microbiomes of all three nematode species were dominated by the phylum Proteobacteria (53%, 70% and 73% for Pm I, Pm II and Pm III, respectively). The phyla Bacteroidetes (10%, 14% and 1.8% for Pm I, Pm II and Pm III, respectively) and Actinobacteria (17%, 6% and 5% for Pm I, Pm II and Pm III, respectively) were the second and third most abundant group of bacteria, which were found in nearly all specimens (17 and 18 of the specimens, respectively). The Verrucomicrobia were present in 5 of the 6 specimens of Pm III with an average relative frequency of 16%, whereas its frequency in Pm I and Pm II was less than 1% and 4%, respectively and in 4 and 2 of the 6 specimens, respectively. The Firmicutes group was present in all 18 specimens in similar frequencies (2.1%, 3.6% and 3.8% in species Pm I, Pm II and Pm III respectively). In total, 79 OTUs were unassigned, but nearly all of them had a relative frequency of less than 1% and their total abundance reached 9.9%. New.ReferenceOTU30 was prominent in Pm I (12 % in the rarefied dataset), but only in one replicate. Within the phylum Proteobacteria, the Gammaproteobacteria dominated the microbiomes of Pm I (82.7%) and Pm II (72.7%) and to a lesser extent the microbiome of Pm III (46.4%) (Fig. 1A) and contained 57 taxa from 22 known families (Fig. 1B). The Alteromonadaceae and Moraxellaceae were amongst the most abundant families shared between the three species and were especially abundant in Pm III (12.6% and 15.5%) (Fig. 1B). The Alphaproteobacteria formed the second most abundant class within the Proteobacteria, and represented 9.6%, 18.1% and 44.2% of the assigned taxa of Pm I, Pm II and Pm III, respectively (Fig. 1A). This group comprised 44 taxa belonging to 15 known families, of which the Caulobacteraceae, Rhodobacteraceae and

Sphingomonadaceae were the most abundant (Fig. 1C). Especially the latter family was much more abundant in Pm III (20.7%) than in Pm I (1.8 %) and Pm II (1.7%) but this was caused by a high abundance in one specimen (175Pm III, Fig. 1C). The Beta, Delta and Epsilon Proteobacteria were only poorly represented, and contained 28, 10 and 2 taxa, respectively.

Within the phylum Actinobacteria, more than 99% of the taxa belonged to the Actinobacteria class, within which 17 families were assigned (Fig. 2A). Two families, the Corynebacteriaceae and the Microbacteriaceae, were prominent in all three nematode species. The high abundance of the Microbacteriaceae in Pm I was mainly caused by a high abundance in a single specimen (145Pm I, Fig. 2A).

Within the phylum Bacteroidetes, two classes encompassed more than 99% of the assigned taxa: the Flavobacteria dominated Pm I and Pm III (75.3% and 81.8%, respectively), while the Cytophagia dominated Pm II (68.0%, versus 23.2% and 17.4% in Pm I and Pm III). Both classes were represented by only two families: the Cytophagia consisted of Cytophagaceae and Flammeavirgaceae (Fig. 2B), the latter being found in very low abundance and in only one specimen of each species; the Flavobacteria consisted of Flavobacteriaceae, Cryomorphaceae and Weeksellaceae, the former being dominant in Pm I, while the Weeksellaceae were abundant in Pm II (Fig. 2B).

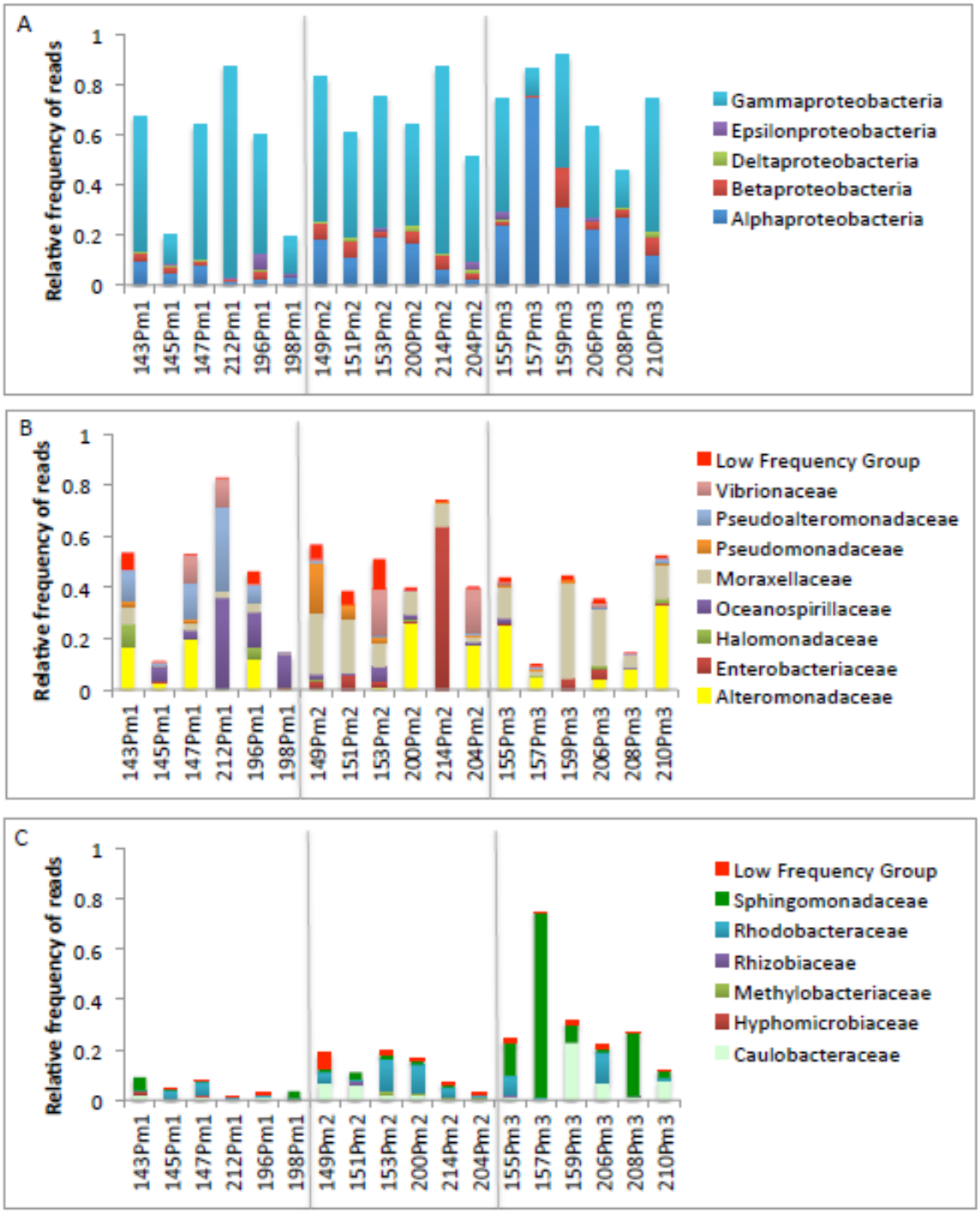



Figure 1: Relative composition of Proteobacteria for each of the 18 nematode specimens. Reads are from the Reverse dataset. A/Class level; B/ Family level Gammaproteobacteria, the eight most abundant taxa are shown, the 14 remaining taxa are pooled in a "Low Frequency Group"; C/ Family level Alphaproteobacteria, the six most abundant taxa are shown, the nine remaining taxa are pooled in a "Low Frequency Group".

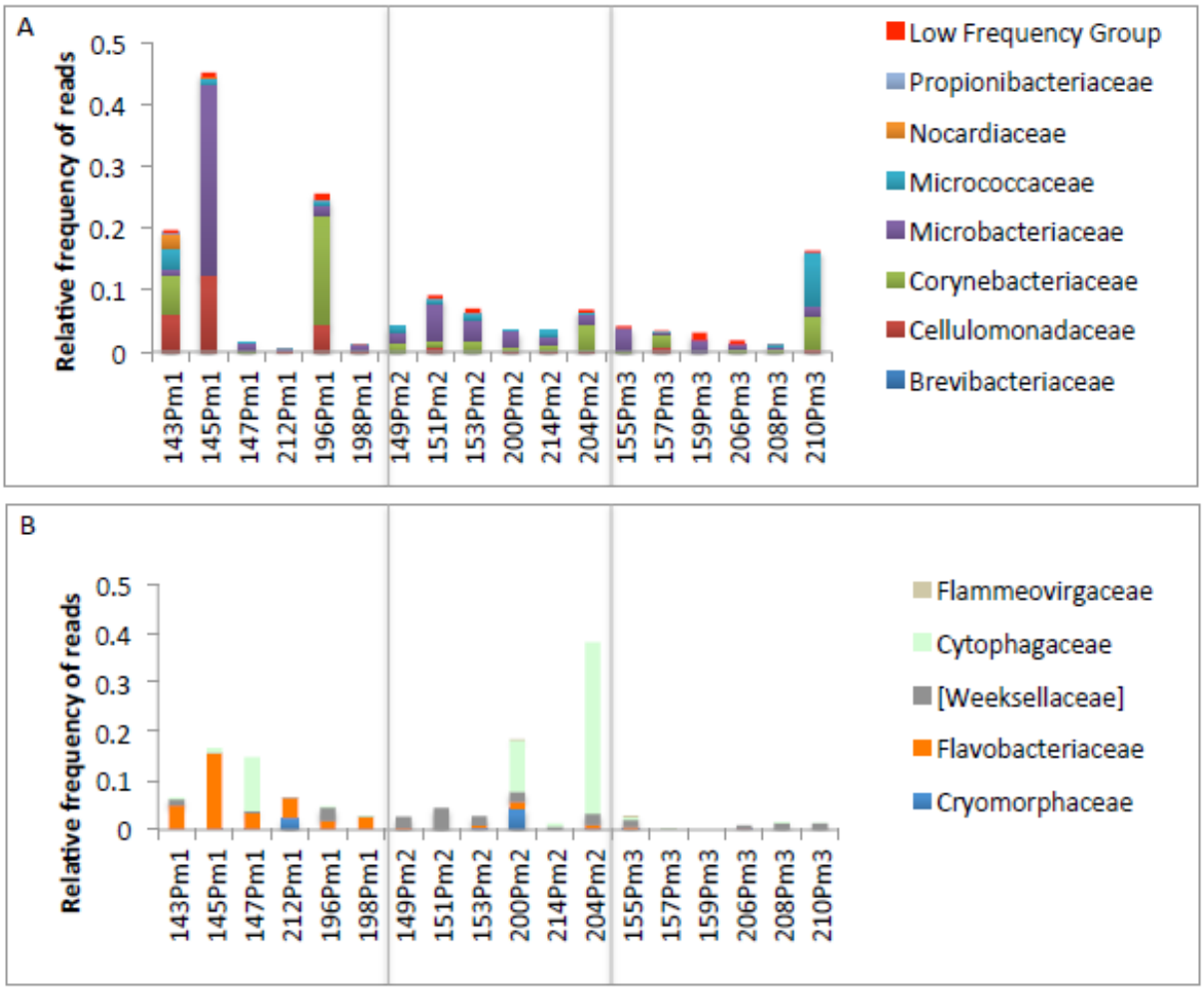



Figure 2: Relative taxonomic composition of bacteria for each nematode specimen at the family level. Reads are from the Reverse dataset. A/ Actinobacteria, the seven most abundant families are shown, the remaining ten families are pooled in a "Low Frequency Group". B/ Bacteriodetes.

*B. Alpha diversity of field specimens*

Rarefaction curves of the number of observed OTUs yielded highly similar results for Forward and Reverse datasets. Curves were still increasing at a sampling depth of 600 sequences per nematode specimen (Fig. 3a, appendix S5). In contrast, the Shannon diversity measure quickly reached a plateau (Fig. 3b, appendix S5), suggesting that many OTUs occur in very low frequencies. This was confirmed by the rank abundance plot, which illustrates that only a few OTUs have relative abundances higher than 0.1, while many OTUs have very low relative abundances (Appendix S6).

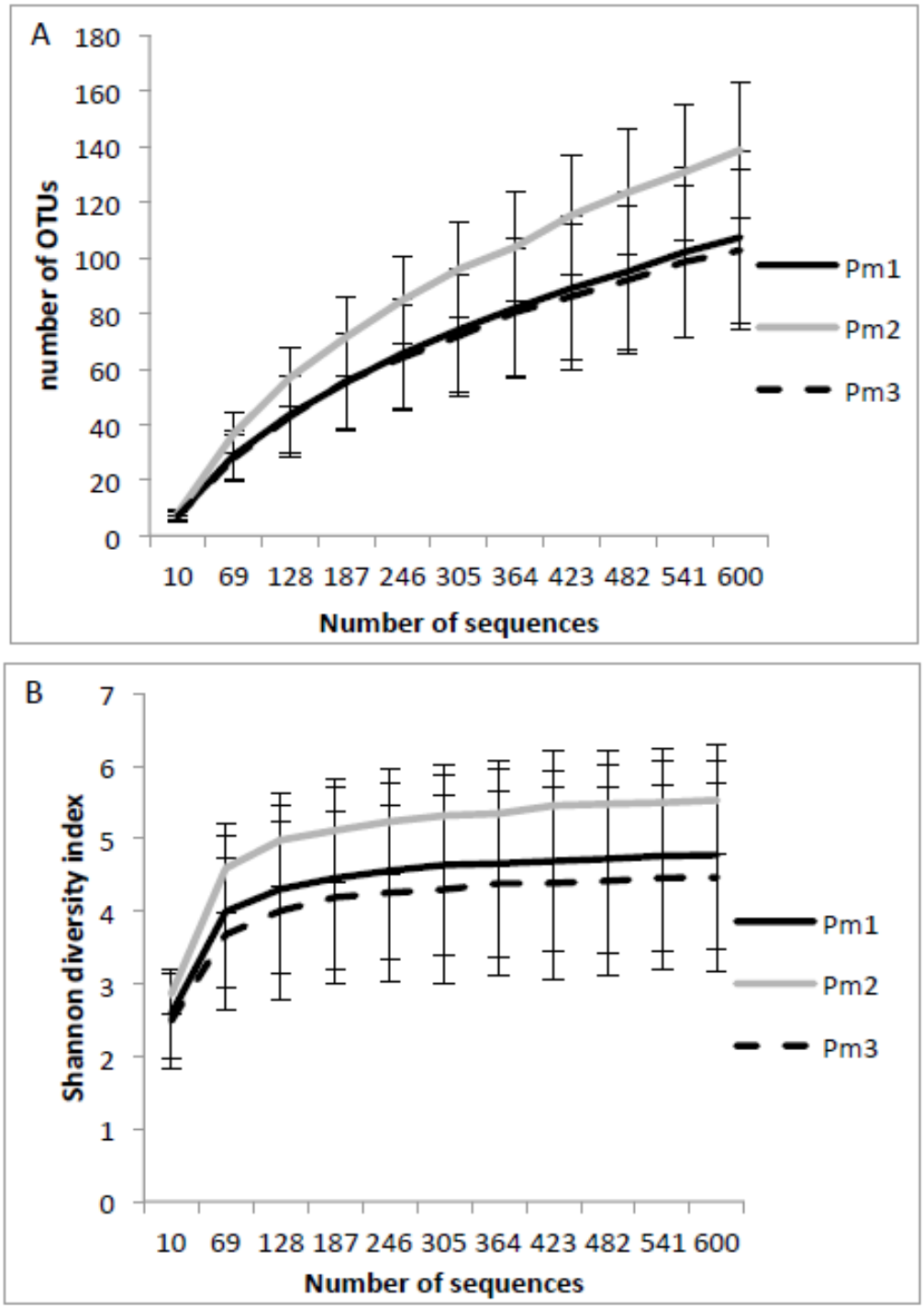



Figure 3: Rarefaction curves of the number of observed OTUs at 97% sequence identity clustering (A) and Shannon index (B) for each species for the Reverse dataset. Error bars were calculated from the variance of the respective parameter drawn in 10 randomizations at each sample size.

*C. Beta diversity of field specimens*

Permanova based on the Generalized Unifrac distances showed significant differences between the microbial communities of the nematode species for both Forward and Reverse datasets and with or without inclusion of rare OTUs (Table 1). Post hoc tests revealed that these differences were situated between Pm I and Pm III, regardless the dataset used. The six specimens within species did, however, show substantial variability (Fig. 4, appendix S5). The non-significant Permdisp results (Table 1) indicated that intraspecific differences were comparable for each of the three species.

Table 1: Summary of the Permdisp and Permanova statistics between the microbiomes of the nematode species Pm I, Pm II and Pm III. Analyses were done on the Forward and Reverse datasets using all OTUs or only those OTUs with relative frequency in the rarefied dataset ≥ 1%. For the pairwise comparisons, significant p-values are indicated in bold.

| | | | All OTUs | | OTUs > 1% | |
|---|---|---|---|---|---|---|
| Forward dataset | | df | F | p value | F | p value |
| PERMDISP | | 2 | 0.51 | 0.61 | 0.75 | 0.49 |
| Overall PERMANOVA | | 2 | 1.79 | **0.007** | 1.76 | **0.04** |
| Pairwise test | Pm1-Pm2 | 1 | 1.55 | **0.03** | 1.34 | 0.19 |
| Pairwise test | Pm1-Pm3 | 1 | 2.21 | **0.008** | 2.65 | **0.016** |
| Pairwise test | Pm2-Pm3 | 1 | 1.61 | 0.062 | 1.37 | 0.23 |
| Reverse dataset | | | | | | |
| PERMDISP | | 2 | 1.73 | 0.211 | 1.69 | 0.22 |
| Overall PERMANOVA | | 2 | 1.62 | **0.001** | 1.90 | **0.012** |
| Pairwise test | Pm1-Pm2 | 1 | 1.40 | **0.032** | 1.46 | 0.11 |
| Pairwise test | Pm1-Pm3 | 1 | 2.11 | **0.002** | 2.88 | **0.004** |
| Pairwise test | Pm2-Pm3 | 1 | 1.36 | 0.074 | 1.50 | 0.13 |

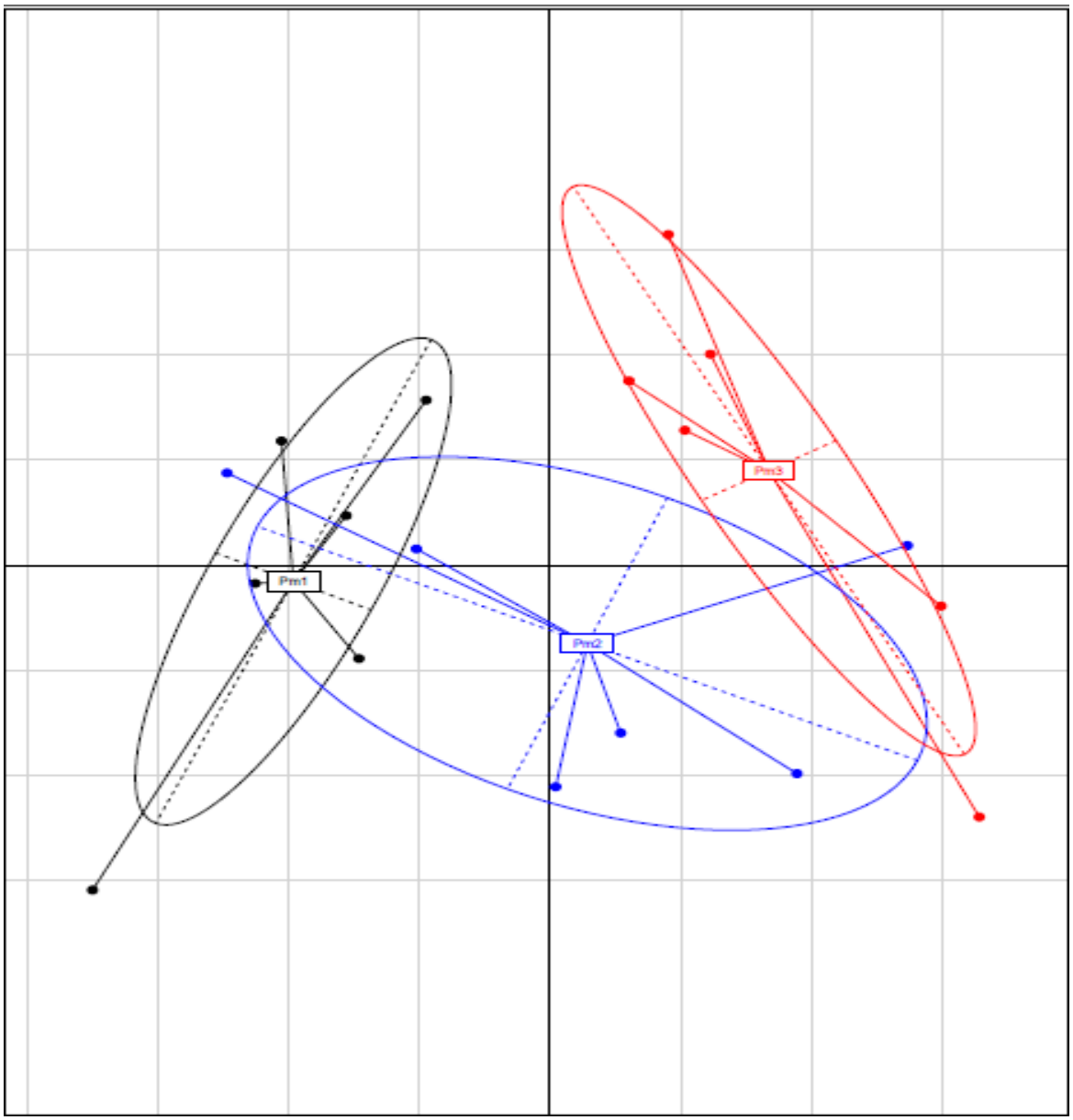



Figure 4: Principal coordinates analysis plot based on Generalized Unifrac distances between 18 nematode specimens after rarefaction at 600 sequences per specimen of the reverse dataset from the 454 platform. Intraspecific distances (distances to the centroid) for Pm I (black), Pm II (blue) and Pm III (red) are encircled (95 % of the inertia of the corresponding group).

*D. The core microbiome of field specimens*

Despite the high number of OTUs observed for each nematode species (see appendix S3), none of them were shared between all 18 specimens. The core microbial community for each species consisted of very few OTUs (5, 5 and 2 OTUs for species Pm I, Pm II and Pm III in the Forward dataset, respectively, and 5, 6 and 4 OTUs for species Pm I, Pm II and Pm III in the Reverse dataset, respectively; see appendix S7). Frequencies of the core communities were overall low in each of the 18 specimens, but 4 and 6 core OTUs of the forward and reverse datasets respectively reached frequencies higher than 1% (Fig. 5). The core communities of species Pm I and Pm II were also present in the other species, while the core community of species Pm III was nearly absent in the two other species. Permanova on the generalized unifrac distances yielded only borderline (non-) significant differences between the three species (Reverse dataset: F= 2.40, p = 0.058; Forward dataset: F= 2.94, p= 0.048), suggesting that the core communities were phylogenetically similar to each other. Small differences in taxonomic composition were however present (Appendix S7). OTU clustering at 99% slightly increased the number of core OTUs (8 vs 5 for Pm I, 6 vs 6 for Pm II and 5 vs 4 for Pm III) which was mainly due to an increase of OTUs identified as Moraxellaceae. Taxonomic composition was very similar to that observed with 97% clustering (Appendix S7).

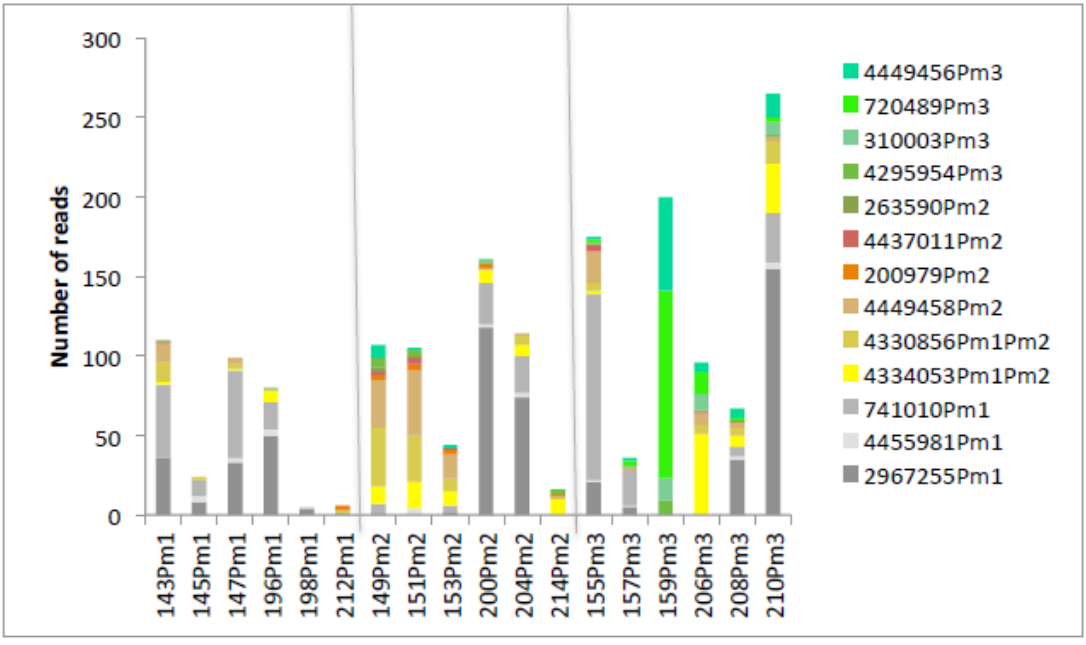



Figure 5: Number of reads assigned to the core OTUs of Pm1, Pm2 and Pm3 in each of the 18 specimens from the rarefied reverse dataset. Legend reflects OTU name followed by the name of the species in which they were the core (e.g. 4334053Pm1Pm2 indicates that OTU 4334053 was present in all six specimens of Pm1 and in all six specimens of Pm2).

*E. Biomarker taxa of the field specimens*

The LeFSe analysis indicated 1, 2 and 6 taxa that significantly differentiated Pm I, Pm II and Pm III respectively and with an LDA score higher than two. The biomarker for Pm I belongs to the genus *Pseudoalteromonas* (OTU4406967). New.ReferenceOTU37 and OTU200979 were identified as biomarker for Pm II and belong to the genus *Microbacterium* and the ordo Saprospirales respectively. The biomarker taxa of Pm III were identified as Verrucomicrobiaceae (New.ReferenceOTU54 and OTU4307243), *Acinetobacter* (OTU4449456), Moraxellaceae (OTU4334053), Caulobacteraceae (OTU310003) and Comamonadaceae (OTU115161) (Appendix S7).

**16S rRNA composition of individual nematode specimens from the food experiment**

To investigate whether the observed differences in the microbiomes of Pm I and Pm III were related to selective feeding, we performed a food experiment in which both species were offered *E. coli* or a diverse bacterial mix as food. The MiSeq protocol generated a much larger number of sequences and OTUs per nematode specimen (Appendix S8) than the 454 protocol. A detailed description of the taxonomic composition of the non-rarefied dataset of the food experiment can be found in appendix S9. The microbiomes of all samples were clearly dominated by Proteobacteria and Bacteroidetes (Fig. 1 in appendix S9). At the family level, the microbiomes of the two food treatments showed some striking differences between each other, but also between species: 1/ within Alphaproteobacteria, the microbiomes of Pm III worms fed the bacterial mixture resembled the bacterial mixture, while the microbiomes of the Pm III worms fed *E. coli* contained a substantial amount of Rhodobacteraceae, which were highly abundant in the Pm III stock cultures (Fig. 6A). In contrast, Pm I worms showed very similar compositions regardless the offered food. 2/ Within Gammaproteobacteria, the microbiomes of Pm I and Pm III fed the bacterial mix were similar to that of the bacterial mix. The microbiomes of Pm I and Pm III worms fed *E. coli* resembled that of the stock cultures of each species (Fig. 6A). Surprisingly, the worms fed *E. coli* were not enriched for Enterobacteriaceae. However, the *E. coli* suspension that was offered to the nematodes in the *E. coli* treatments was dominated by Enterobacteraceae (Fig. 6B). 3/ Within the Bacteroidetes, all Pm III worms were dominated by Saprospiraceae, the dominant family of the bacterial mix. Abundances of this family were higher in the Pm III worms fed the bacterial mix than those that had been fed *E. coli*. For Pm I, taxonomic composition of both food treatments was comparable (Fig. 6A).More details on the taxonomic composition can be found in Appendix S9.

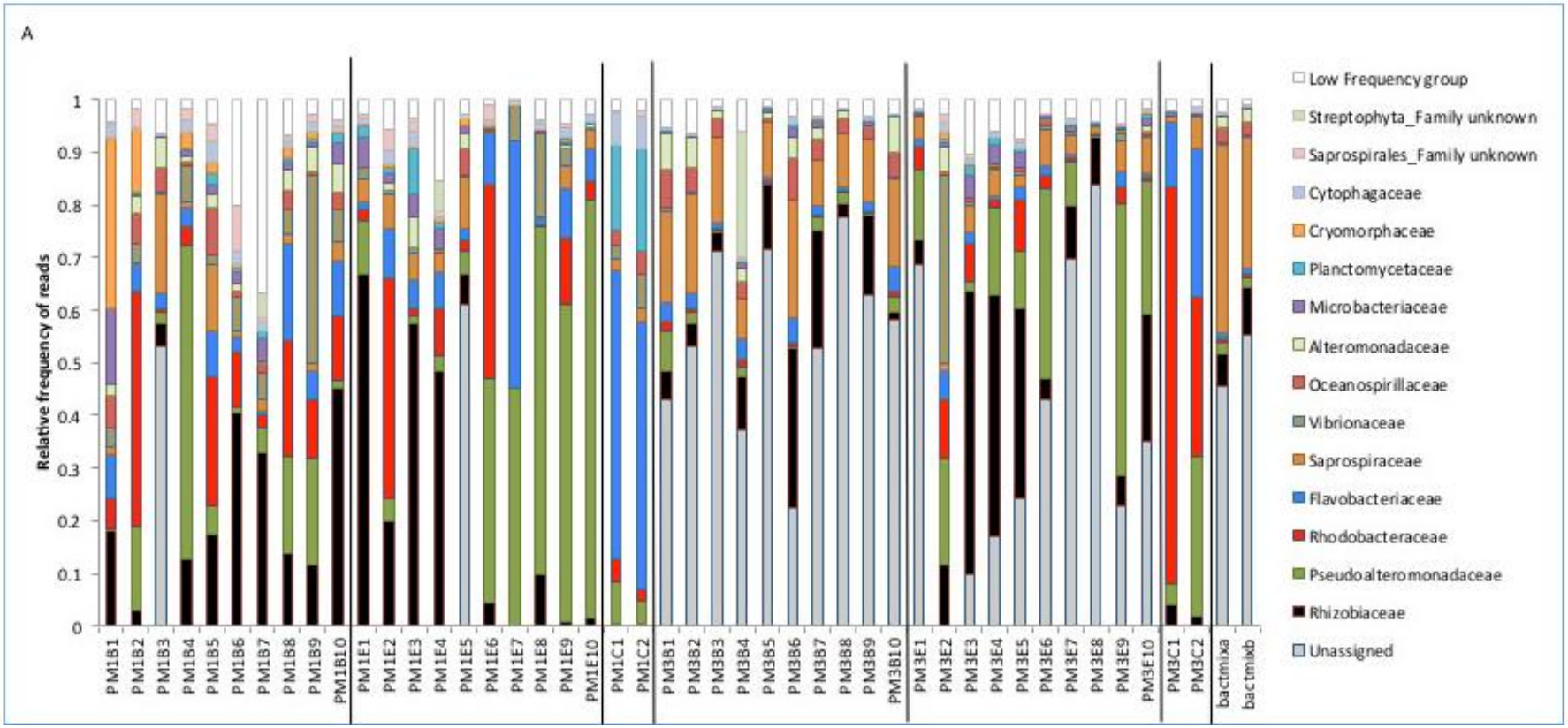



Figure 6A: Taxonomic assignment of MiSeq reads at the family level for the food experiment. For the food experiment, the 15 most abundant families are shown, the remaining families are pooled in a "Low Frequency Group". Pm1B1-10: 10 biological replicas of Pm1 fed the bacterial mixture; Pm1E1-10: 10 biological replicas of Pm1 fed *E. coli*; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; Pm3B1-10: 10 biological replicas of Pm3 fed the bacterial mixture; Pm3E1-10: 10 biological replicas of Pm3 fed *E. coli*; Pm3C1-2: two biological replicas of the agar from Pm3 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. Vertical grey lines denote the different food treatments, stock cultures and bacterial mix.

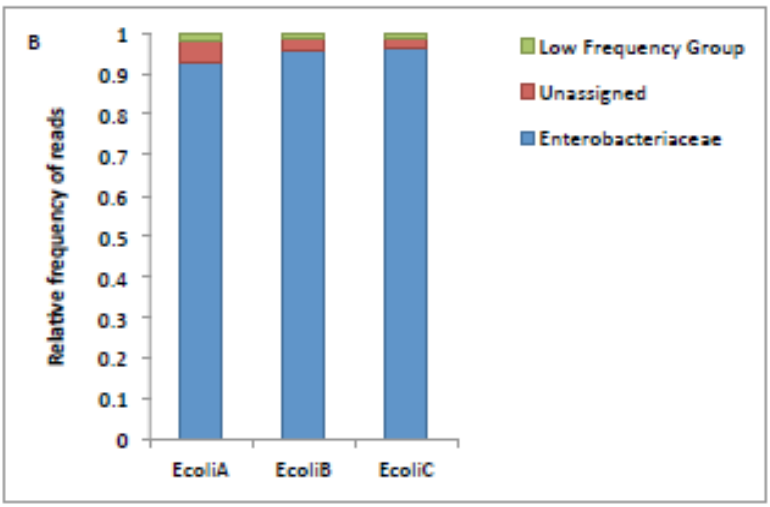



Figure 6B: Taxonomic assignment of MiSeq reads at the family level for three biological replicas of the *E. coli* suspension. EcoliA-EcoliC: three biological replicas of the *E. coli* suspension. The "Low Frequency Group" contains 16 families.

*A. Alpha diversity of specimens from the food experiment*

The average number of OTUs observed in the nematodes (regardless species) fed the bacterial mix was similar to that in those fed *E. coli* (Kruskal-Wallis: df = 6, p = 0.08). Patterns of species diversity and richness were very similar to the data on the field specimens: the number of OTUs was still increasing at a sampling depth of 41000 sequences per treatment, the Shannon diversity measure quickly reached a plateau, and the rank abundance plots again show that many OTUs have very low relative abundances (Appendix S9, S10). Four OTUs were highly abundant in the Pm I specimens from the *E. coli* treatment and are thus likely to be part of the microbiome sensu stricto: *Pseudoalteromonas* (ca 98 000 reads), *Agrobacterium* (ca 69 000 reads), Unassigned (ca 57 000 reads) and *Winogradskyella thalassocola* (ca 32 000 reads). When blasted in Genbank, the unidentified OTU was most similar to an uncultured bacteria from a water cave (accession number FJ604748.1). The most highly abundant Pm3E OTU (ca 150 000 reads) was the same unidentified OTU as for Pm1E.

*B. Beta diversity of specimens from the food experiment*

Permanova based on the Generalized UniFrac distances of the four food x species treatments (Pm1B, Pm1E, Pm3B, Pm3E) showed significant differences between food (pseudo $F_{1,39}$= 3.42; p=0.005) and species (pseudo $F_{1,39}$= 10.97; p=0.001). The interaction between food and species was only just significant (pseudo $F_{1,39}$= 2.02; p=0.049). Pairwise comparisons were all significant, except for Pm1B and Pm1E (Table 2). The principal coordinates analysis showed that species is the most important grouping factor (Fig. 7). Within each species, Pm I showed high intraspecific variability in both food treatments, while intraspecific variability for Pm III was much lower in the treatment where they were offered a bacterial mix. Homogeneity of dispersions was not achieved (p>0.05) for factor species and the interaction of species with food (Table 2). Multivariate dispersions differed only within the B treatment between Pm I and Pm III (pairwise Permdisp: $F_{1,18}$ = 13.88 , p-=0.001), reflecting the low variation within Pm III in this treatment.

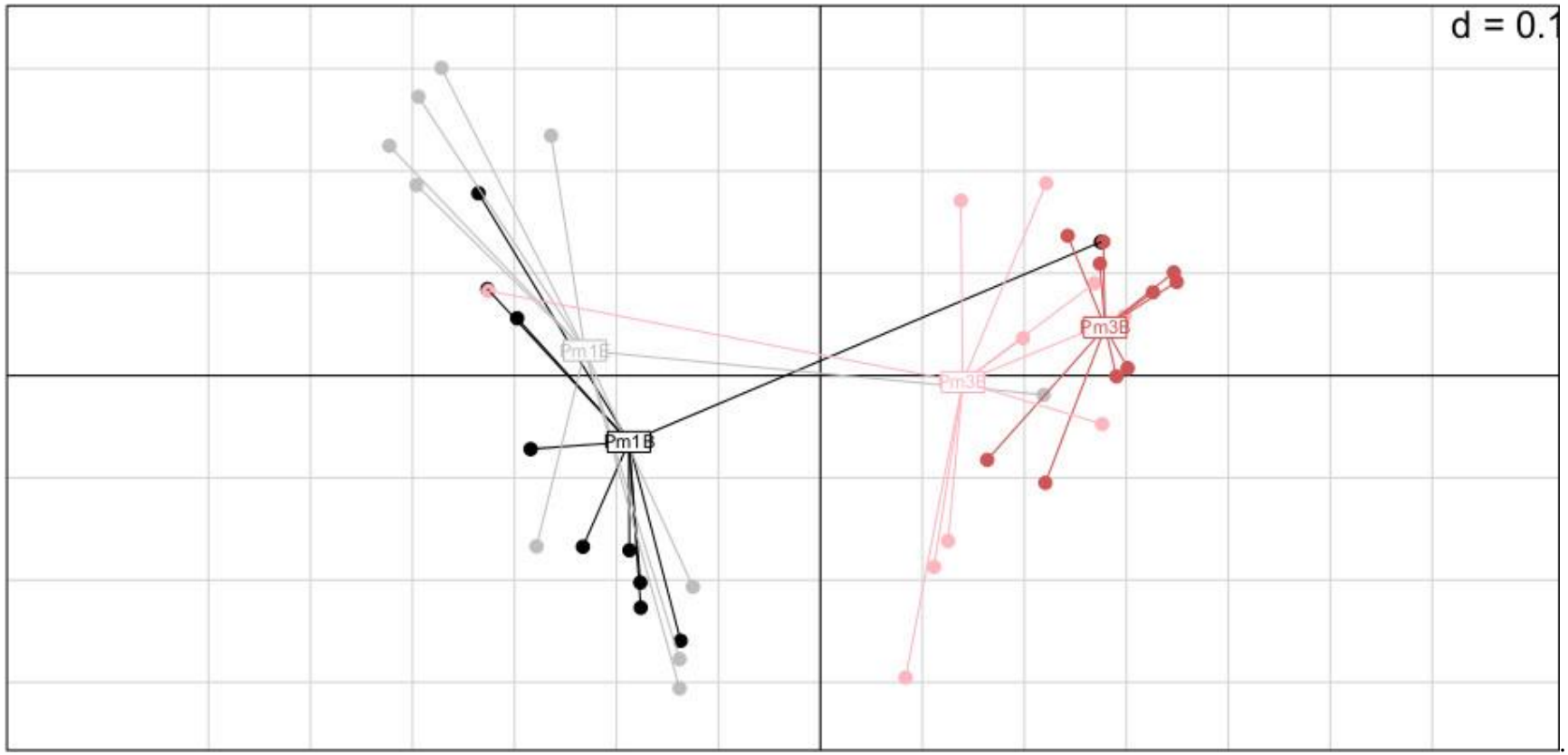



Figure 7: Principal coordinates analysis plot of the Generalized Unifrac distances for the two species (Pm1 black/grey and Pm3 red/pink) and the two food treatments. E = *E. coli* (grey/pink) and B = bacterial mixture (black/red).

*C. The core microbiome of specimens from the food experiment*

Similar to the results of the field specimens, the fraction of OTUs shared between all specimens was very low. In total, 41 OTUs were shared between all 46 samples of the food experiment. The core of the Pm I bacterial mixture treatment had 157 OTUs and the Pm III bacterial mixture treatment had 261 core OTUs. The number of core OTUs was lower for the *E.coli* treatment: 85 core OTUs were present in Pm I and 178 for Pm III. The core of all 20 Pm III individuals contained 77 OTUs, while Pm I had 52 OTUs shared among all 20 specimens. Permanova on UniFrac distances showed that food (pseudo-$F_{1,39}$=3.59,p=0.008), species (pseudo-$F_{1,39}$=16.56, p=0.001) and the interaction food*species (pseudo-$F_{1,39}$=2.46,=0.043) were significant. All pairwise comparisons were significant, except for the two food treatments of Pm I (Table 2).

Table 2: Summary of the Permdisp and Permanova statistics between the microbiomes of the four food experiment treatments (Pm1B, Pm1E, Pm3B and Pm3E) for the dataset containing all OTUs and for the core OTUs. For the pairwise comparisons, significant p-values are indicated in bold.

| | | | All OTUs | | Core Genome | |
|---|---|---|---|---|---|---|
| Food experiment | | df | Pseudo-F | p value | Pseudo-F | p value |
| PERMDISP | species | 2 | 9.04 | **<0.001** | 7.11 | **0.011** |
| | food | 1 | 2.94 | 0.095 | 1.57 | 0.22 |
| | species*food | 2 | 6.80 | **<0.001** | 6.65 | **0.001** |
| Overall PERMANOVA | species | 2 | 10.97 | **0.001** | 16.56 | **0.001** |
| | food | 1 | 3.10 | **0.005** | 3.59 | **0.008** |
| | species*food | 2 | 2.02 | 0.049 | 2.46 | 0.043 |
| Pairwise test | Pm1B - Pm1E | 1 | 1.65 | 0.236 | 1.62 | 0.13 |
| Pairwise test | Pm3B - Pm3E | 1 | 3.98 | **0.004** | 5.50 | **0.001** |
| Pairwise test | Pm1B - Pm3B | 1 | 8.78 | **0.004** | 14.71 | **0.001** |
| Pairwise test | Pm1E - Pm3E | 1 | 4.81 | **0.004** | 6.1 | **0.002** |

*D. Biomarker taxa of specimens from the food experiment*

For Pm I, 433 OTUs were identified as biomarkers, while 208 OTUs were identified as biomarker for Pm III. Taxonomic assignment of many OTUs was only achieved at the class level and 52 OTUs of the Pm III biomarker taxa had no taxonomic assignment at all (Appendix S11). The biomarker OTUs that were identified up to family level belonged to the Flavobacteriaceae, Rhodobacteraceae, Alteromonadaceae, Pseudoalteromonadaceae and Vibrionaceae for both species, with an additional two families for the biomarker taxa of Pm I (Phyllobacteriaceae and one unidentified family of the ordo Saprospirales). The complete list of biomarker OTUs for Pm I and Pm III with their taxonomic assignment can be found in appendix S11.

**SEM and light microscope pictures**

SEM pictures revealed that the cuticle of the cryptic nematode species contained only very few bacteria, which were mainly located in the mid body region for the females, and in the tail region for the males (see appendix S12). The morphology of the attached bacteria was quite uniform, suggesting a very low taxonomic diversity of the epibionts. The digital pictures that were taken from the sequenced specimens' seconds before transferring them into the WLB further support that the bacterial densities and diversity on the cuticle of the three rhabditid nematodes were low.

## Discussion

### The nematode microbiome is highly diverse and species-specific

Our data show that the bacterial community associated with the *Litoditis* specimens contains at least 85 OTUs for the field specimens (Appendix S3). Most OTUs were present in very low frequency. Even under laboratory conditions and with *E. coli* as a food source, a high diversity was associated with the nematode specimens (lowest number: 1118 OTUs, Appendix S8). Applying the MiSeq protocol to the field specimens would very likely result in an even higher diversity than observed in the laboratory specimens. The microbiomes of the field specimens and cultured nematodes are not directly comparable because two different sequencing platforms (454 vs. Illumina platforms) and primer sets were used to generate sequence data which may introduce taxonomic and technical biases in terms of the microbial community recovered.

Despite the high number of bacterial OTUs associated with the field nematode specimens, only 2 - 6 OTUs were found in all six specimens of a particular species, and not a single OTU was found in all 18 specimens (see appendix S7). This was also true for the food experiment, in which 52 OTUs were shared among the 20 Pm I specimens and 77 OTUs were shared amongst the 20 Pm III specimens. The frequency of the core microbiome was very low, and although six core OTUs obtained a frequency higher than 1% in the rarefied dataset, their abundance varied substantially between individuals (Fig. 5). Bacterial strains that are present in the core microbiome of a particular nematode species and that are absent in the other species can potentially confer an adaptation to the environment for that particular nematode species. Moreover, if such core OTUs are also present in the other nematode species than the species for which it is a core OTU, its abundance should be significantly different between nematode species. In other words, it would be identified as biomarker in the LeFSe analysis. Three core OTUs of Pm III were completely absent in Pm I when clustering at 97% for the reverse dataset (Appendix S7): OTU310003 (Caulobacteraceae), OTU720489 (*Acinetobacter*) and OTU4449456 (*Acinetobacter*). They may thus be involved in mediating different tolerances - by contributing to the fitness of the host by for instance influencing development, reproduction, metabolism or lifespan (Cabreiro & Gems, 2013)- to environmental conditions for Pm I and Pm III. Two of these Pm III core OTUs were also present in Pm II (OTU310003 and OTU4449456) and were identified as biomarkers for Pm III by the LeFSe analysis, suggesting that members of Caulobacteraceae and *Acinetobacter* may be involved in differential abiotic tolerances for Pm III. All Pm II core OTUs were

present in the two other species, and only one was identified as a biomarker for Pm II: OTU200979 (*Microbacterium*). This OTU may thus potentially be involved in generating tolerance to abiotic conditions for Pm II. Laboratory experiments show that Pm I performs less well at higher temperatures, while population development of Pm III was lower at lower temperatures (De Meester, et al., 2015a; chapter III). This corresponds with the prevalence of Pm III during warmer seasons and to its near-absence during colder seasons (Derycke, et al., 2006). Pm II has a pan European distribution and appears to be a generalist as it is found in habitats that differ substantially in temperature and salinity (Derycke, et al., 2008b). The microbiome '*sensu stricto*' may perform a critical role in the physiological adaptations to such environmental changes.

**Sympatric, cryptic nematode species show differences in resource use**

We hypothesized that the differences in the microbiomes '*sensu lato'* between the nematode species were linked to differential resource use, as all three species are bacterivorous. We expected to find many more OTUs in the worms that had been feeding on the bacterial mix compared to those that had been fed *E. coli*. This appeared not to be the case, but there was a significant food effect (Table 2) on the microbiome (dependent on species), indicating that bacteria were differentially consumed by the worms in the two food treatments. The similar number of OTUs observed in both food treatments may indicate that the worms only fed on a small number of OTUs present in the bacterial mix. Yet, the taxonomic composition of the worms fed on the bacterial mix was quite diverse and resembled the one of the bacterial mix (Fig. 6A: mostly true for Pm III (see further)). The stock cultures of both worms contained a large number of OTUs (1996 and 1301 for Pm I and Pm III respectively, appendix S8) indicating that the microbiome *sensu stricto* is highly diverse and that several bacterial strains of this microbiome are able to grow on the agar. The Pm I and Pm III microbiomes from the *E. coli* treatment shared 1271 and 1135 OTUs with the Pm I and Pm III culture microbiome, respectively. Consequently, the potential food of the worms in the *E. coli* treatment was probably as diverse as the bacterial mix (which contained 2496 OTUs versus 552 OTUs for the *E. coli* suspension, appendix S8). OTUs showing higher abundances in the cultures did not result in a higher abundance in the microbiome and vice versa. Moreover, the microbiomes of specimens fed with *E. coli* resembled the one of the stock cultures (Fig, 6A), and their intestinal colour clearly indicated that they were actively feeding to a similar extent as the specimens in the bacterial mix treatment, adding support to the idea that the worms in the *E. coli* treatments had a much more diverse food source than anticipated.

Surprisingly, we did not find an increase of Enterobacteraceae in the specimens fed *E. coli*. Yet, the *E. coli* suspension was clearly dominated by Enterobacteraceae (Fig. 6B), providing evidence that our methodological approach was able to identify the *E. coli* sequences. The *E. coli* source consisted of frozen and thawed *E. coli* cells, and provided as such a "soup" rich of nutrients instead of metabolically active cells. Add-back experiments have demonstrated that *C. elegans* requires metabolically active cells for normal development and fecundity (Lenaerts, et al., 2008). Tracer experiments with *Litoditis* showed that radioactive labels were only present in the worms when fed labelled (unidentified) bacteria, while such a radioactive signal was absent when the worms were offered the growth medium of that same bacterial mix without cells despite the fact that this medium was much more heavily labelled than the bacterial cells (Moens, unpublished data). This suggests that the nutrient rich "soup" provided by the *E. coli* suspension can stimulate extensive growth of other bacteria from the worm microbiome (both from gut and cuticle) and that the soup itself was not ingested by the worms.

The food experiment further showed that the microbiome of Pm I did not differ according to food type, while that of Pm III did. This result can be explained by two non-mutually exclusive scenarios: 1/ the Pm III microbiome '*sensu stricto*' (Pm3E) differs considerably from the bacterial mixture while the Pm I microbiome '*sensu stricto*' (Pm1E) is similar to the bacterial mixture. Feeding of Pm III on the bacterial mix would then lead to significant differences between Pm3E-Pm3B but not between Pm1E-Pm1B. Comparison of the number of OTUs shared between the *E. coli* fed specimens and the bacterial mix do not support this hypothesis, since Pm III specimens typically show a higher number of shared OTUs with the bacterial mix than Pm I specimens (Appendix S13); 2/ the two species show different feeding behaviours with Pm III feeding more selectively on a smaller portion of the bacterial mixture, while Pm I feeds on a much wider range of bacterial strains from the mixture. This hypothesis is supported by the larger variability between individual Pm I specimens that were fed the bacterial mix compared to the much smaller interindividual variability in Pm III (PCoA plot, Fig. 8; significant pairwise Permdisp) and by the higher number of biomarker taxa identified in Pm I compared to Pm III (Appendix S11), indicating that Pm III is a much more selective feeder than Pm I. We also found a significant species effect (Table 2), suggesting that Pm I and Pm III were feeding on different bacterial species. Since the Pm I and Pm III nematodes from the food experiment have been kept for several generations under controlled abiotic conditions, the biomarker taxa revealed by the LeFSe analysis are likely

linked to differential resource use of the two species. The individual differences in bacterial diet cannot be linked to particular life stages or certain ecological morphs since we only selected adult specimens for our population genetic analysis (Derycke, et al., 2006). Observations on the feeding behaviour of living *Litoditis "marina"* specimens showed that the size of the prey forms an important filter for ingestion (Tietjen & Lee, 1975; Moens & Vincx, 1997), and the buccal cavity of Pm III specimens is smaller than that of Pm I specimens (Derycke, et al., 2008a) suggesting that size selection may be one aspect contributing to differences in selectivity. We cannot exclude that the four OTUs potentially involved in adaptation to abiotic conditions are linked to resource use, but *Microbacterium* was present in all three nematode species, and also different *Acinetobacter* OTUs were found in all three nematode species, suggesting that these types of bacterial strains can be ingested by all three species and that size selection through feeding may not be an important mechanism to explain the different abundances of these core OTUs. Instead, the high variability among individuals implies that there are constraints in resource use that prevent individuals from using the whole range of available resources. These constraints may act at the individual level (e.g. uptake ability, morphology, behaviour), but probably more so at the population level, where high intraspecific competition can increase individual niche specialization (Svanbäck & Bolnick, 2007). Intraspecific competition has been observed in all three species (De Meester, et al., 2015b; chapter VII) and individual niche specialization can increase the niche breadth of the total population (Bolnick, et al., 2007). This agrees well with the high diversity of the microbiomes observed in each of the three species, and can affect interspecific interactions, since niche overlap between species is likely to increase with increased niche width.

**Niche partitioning between cryptic species can partially explain their coexistence**

The nematode microbiomes were dominated by Alpha- and Gammaproteobacteria, Bacteroidetes and Verrucomicrobia which are the dominant groups found on *Fucus vesiculosus (*Lachnit, et al., 2011), the habitat from which the nematode specimens were isolated. The microbiomes of Pm I and Pm III from the field were clearly different from each other, and the food experiment shows that these differences are linked both to the feeding activity of the species but also to the presence of a nematode species-specific microbiome. Pm I and Pm III specimens more often co-occur in the field than Pm I with Pm II or than Pm III with Pm II (Appendix S1). These data agree well with the ecological theory of resource partitioning, where species can coexist when they are using different resources (MacArthur

& Levins, 1967). However, if resource partitioning would be the only driver for coexistence of these cryptic species, we would expect to find Pm I coexisting with Pm III throughout the year, which is not the case (Derycke, et al., 2006). Coexistence of species is also governed by their common responses to environmental changes (Chesson, 2000b; Leibold & McPeek, 2006) and the microbiome may perform a critical role in the physiological adaptations to such environmental changes and hence in the fitness of the nematode hosts. Dedicated attempts (using repeated transfer of worms through mixtures of antibiotics and even incorporating antibiotics in the stock culture media for several subsequent generations) at removing bacteria other than the *E. coli* supplied as food failed (P. Gilarte, unpublished data), suggesting a tight association between nematodes and (components of) their microbiomes. Fitness differences imply that differential responses to abiotic environmental variability can also have stabilizing effects on the coexistence between cryptic nematode species. In addition, the ephemeral nature of the *Fucus* habitat on which the species live also induces strong variability in the environment. The coexistence of Pm I and Pm III is therefore likely to be determined by both resource partitioning and differential responses to abiotic changes. Although microbiome differentiation was less straightforward between Pm I and Pm II, phylogeographic data revealed that Pm II has a more widespread distribution than the two other species, suggesting it has a broader 'abiotic' niche than the other species. The microbiome of Pm II was also not differentiated from either of the two other species.

**Methodological considerations**

Our understanding of the degree of resource selectivity in nematode feeding behaviour is generally very poor: several laboratory experiments have demonstrated a high capacity to select among even very similar food items (Moens, et al., 1999), but reliable approaches to study such detailed resource selectivity under more natural conditions have been lacking. Moreover, stable isotope and other approaches which measure food absorption usually require pooling of individuals for a single analysis (Carman & Fry, 2002).

Our approach complements others, but provides a substantial advance compared to any previous work on resource utilization of free-living nematodes or other microscopic eukaryotes by characterizing the complete bacterial community of individual specimens of three nematode species. The marker gene survey approach used here allows to assess selective feeding behaviour of single nematode specimens, which has not been possible with methods widely used to assess resource use (e.g. stable isotope analysis). However, our results also show the presence of a highly diverse endosymbiont community that differs

substantially among individuals. Our morphological investigation of the bacteria on the cuticula detected only few bacterial morphotypes suggesting that most of the microbiome is located inside the body of the worm (see appendix S12).

**Conclusions**

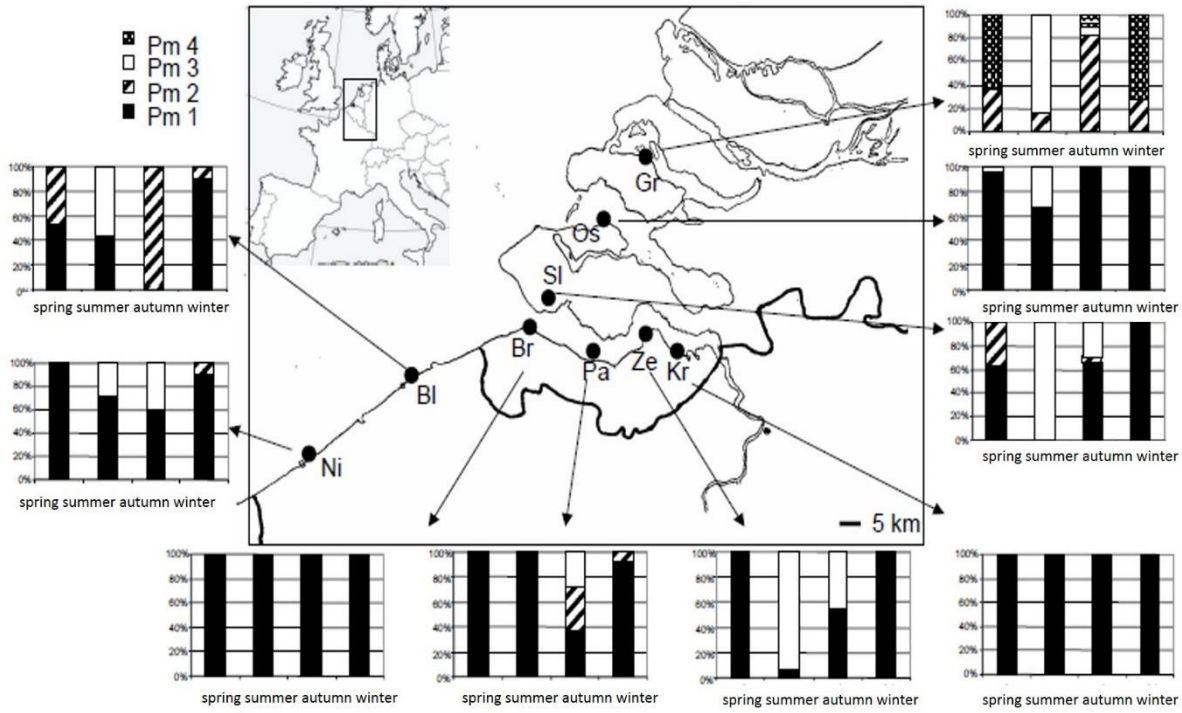
The natural bacterial communities of sympatrically distributed cryptic nematode species are highly diverse and show pronounced intraspecific diversity. The species-specific microbiomes may play a role in the different tolerances of the nematode species to abiotic conditions. Importantly, the differences in selective feeding of morphologically similar nematode species may have a cascading effect on the microbial community and on the functioning of the whole decomposition system, as alterations in microbial communities can alter mineralization of organic matter (Nascimento, et al., 2012). Consequently, cryptic diversity may have hitherto unpredicted consequences for biodiversity-ecosystem functioning relationships in the marine benthos

## Acknowledgements

We gratefully acknowledge John Gaspar for his help in denoising the 454 GS FLX sequence data, and Marjolein Couvreur for generating the SEM pictures. S.D. is funded by the Belgian Science Policy Office (Belspo). This research was financially supported through the F.W.O. projects 3G019209 and 3G038715, and by Ghent University through the BOF project 01GA1911W. We are thankful for the valuable comments of three anonymous referees which greatly improved a previous version of this manuscript.

## Supplementary information

**Appendix S1:** Field distribution of four cryptic *Litoditis "marina"* species (Pm1, Pm2, Pm3 and Pm4) in the Scheldt estuary in The Netherlands in four consecutive seasons. Figure adapted from Derycke, et al. (2006).




Distribution of 4 lineages of *Litoditis "marina"* (Pm I, Pm II, Pm III and Pm IV) inferred from mitochondrial COI data along the Belgian coastline and the Scheldt estuary in The Netherlands. For each location, a stacked column graph indicates the percentage of each lineage occurring in spring 2003, summer 2003, autumn 2003 and winter 2004. Note the small proportion of Pm III during winter 2004 in Br. Sample abbreviations: Ni = Nieuwpoort, Bl = Blankenberge, Br = Breskens, Pa = Paulina, Ze = Zeedorp, Kr = Kruispolderhaven, Sl = Sloehaven, Os = Oosterschelde, Gr = Grevelingen. Figure taken from Derycke, et al., 2006.

**Appendix S2:** Primer sequences used to amplify the 16S rRNA gene of 18 *Litoditis "marina"* specimens in two runs on 1/8$^{th}$ of a plate of the 454 GS FLX Titanium system. Adaptor, midtag and primer sequences for the forward and reverse datasets are given.

| Run1: 9 samples were sequenced on 1/8th of a plate using the 454 platform | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | PCR code | Forward primer | Key | MID | Primer | Reverse primer | Key | MID | Primer |
| PP2085 | B143 | RL17_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | AGTCGTACACT | AACGCGAAGAACCTTAC | RL17_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTGTAGGACT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2086 | B145 | RL18_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | AGTGTAGTAGT | AACGCGAAGAACCTTAC | RL18_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACTACTAGACT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2087 | B147 | RL19_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | ATAGTATACGT | AACGCGAAGAACCTTAC | RL19_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGTATAGTAT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2059 | B149 | RL20_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CAGTACGTACT | AACGCGAAGAACCTTAC | RL20_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTACGTGCTG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2060 | B151 | RL21_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGACGACGCGT | AACGCGAAGAACCTTAC | RL21_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGCGTGGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2061 | B153 | RL22_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGACGAGTACT | AACGCGAAGAACCTTAC | RL22_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTACTGGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2074 | B155 | RL23_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGATACTACGT | AACGCGAAGAACCTTAC | RL23_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGTAGTGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2067 | B157 | RL24_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGTACGTCGAT | AACGCGAAGAACCTTAC | RL24_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ATCGACGGACG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2095 | B165 | RL25_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CTACTCGTAGT | AACGCGAAGAACCTTAC | RL25_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACTACGGGTAG | CGGTGTGTACAAGGCCCGGGAACG |

| Run2: 9 samples were sequenced on 1/8th of a plate using the 454 platform using the same primers as for the first run | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PP2054 | B212 | RL17_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | AGTCGTACACT | AACGCGAAGAACCTTAC | RL17_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTGTAGGACT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2092 | B196 | RL18_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | AGTGTAGTAGT | AACGCGAAGAACCTTAC | RL18_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACTACTAGACT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2103 | B198 | RL19_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | ATAGTATACGT | AACGCGAAGAACCTTAC | RL19_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGTATAGTAT | CGGTGTGTACAAGGCCCGGGAACG |
| PP2058 | B200 | RL20_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CAGTACGTACT | AACGCGAAGAACCTTAC | RL20_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTACGTGCTG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2076 | B214 | RL21_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGACGACGCGT | AACGCGAAGAACCTTAC | RL21_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGCGTGGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2098 | B204 | RL22_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGACGAGTACT | AACGCGAAGAACCTTAC | RL22_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | AGTACTGGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2077 | B206 | RL23_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGATACTACGT | AACGCGAAGAACCTTAC | RL23_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACGTAGTGTCG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2093 | B208 | RL24_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CGTACGTCGAT | AACGCGAAGAACCTTAC | RL24_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ATCGACGGACG | CGGTGTGTACAAGGCCCGGGAACG |
| PP2096 | B210 | RL25_A_16S_968F | CGTATCGCCTCCCTCGCGCCATCAG | CTACTCGTAGT | AACGCGAAGAACCTTAC | RL25_B_16S_1401R | CTATGCGCCTTGCCAGCCCGCTCAG | ACTACGGGTAG | CGGTGTGTACAAGGCCCGGGAACG |

**Appendix S3**: Summary of the sequence data from the two 454 runs performed on 18 fieldspecimens

| | Forward sequences | | | | Reverse sequences | | | |
|---|---|---|---|---|---|---|---|---|
| Samples | raw sequences | sequences after denoising | #OTU_>1 | Good's coverage | raw sequences | sequences after denoising | #OTU_>1 | Good's coverage |
| Pm1_143 | 998 | 937 | 144 | 0.91 | 959 | 891 | 159 | 0.91 |
| Pm1_145 | 14004 | 12995 | 352 | 0.92 | 13896 | 13238 | 473 | 0.93 |
| Pm1_147 | 8285 | 7492 | 357 | 0.88 | 8039 | 7304 | 398 | 0.88 |
| Pm2_149 | 1524 | 1420 | 169 | 0.94 | 1459 | 1374 | 193 | 0.90 |
| Pm2_151 | 699 | 626 | 99 | 0.95 | 1072 | 1011 | 162 | 0.91 |
| Pm2_153 | 4345 | 4134 | 321 | 0.86 | 3998 | 3803 | 370 | 0.85 |
| Pm3_155 | 2697 | 2567 | 263 | 0.86 | 2581 | 2467 | 265 | 0.86 |
| Pm3_157 | 10024 | 9700 | 240 | 0.93 | 11039 | 10829 | 275 | 0.92 |
| Pm3_159 | 839 | 802 | 85 | 0.95 | 682 | 643 | 97 | 0.95 |
| | 43415 | 40673 | 1098 | | 43725 | 41560 | 1295 | |
| Pm1_212 | 22891 | 20564 | 401 | 0.92 | 27557 | 25068 | 513 | 0.92 |
| Pm1_196 | 4755 | 4497 | 291 | 0.88 | 5436 | 5125 | 297 | 0.89 |
| Pm1_198 | 3909 | 3734 | 89 | 0.97 | 4660 | 4473 | 140 | 0.96 |
| Pm2_200 | 3687 | 3430 | 248 | 0.90 | 3502 | 3254 | 297 | 0.90 |
| Pm2_214 | 2044 | 1958 | 116 | 0.94 | 7174 | 6990 | 270 | 0.91 |
| Pm2_204 | 3423 | 3014 | 223 | 0.90 | 4085 | 3676 | 277 | 0.88 |
| Pm3_206 | 1022 | 973 | 111 | 0.93 | 2028 | 1927 | 183 | 0.92 |
| Pm3_208 | 2005 | 1932 | 109 | 0.95 | 2883 | 2782 | 160 | 0.93 |
| Pm3_210 | 2566 | 2419 | 213 | 0.90 | 2348 | 2252 | 224 | 0.91 |
| | 46302 | 42521 | 1036 | | 59673 | 55547 | 1354 | |

Summary of the number of sequences and OTUs obtained after quality filtering using the open reference OTU picking workflow for each nematode specimen ('Samples'). Forward and Reverse sequences were analysed separately. 'Raw sequences': number of sequences obtained without any filtering. 'Sequences after denoising': number of sequences retained in the dataset after filtering and denoising with FlowClus and removal of chimera sequences. '#OTU_>1': the number of OTUs that had more than one sequence in the whole dataset. 'Good's coverage' is calculated per sample. Top 9 specimens are from the first sequencing run, the bottom 9 specimens are from the second sequencing run.

**Appendix S4:** Summary of the analyses to investigate variability between the technical replicates.

To explore the extent of PCR bias, we have labelled the technical replicates from three randomly chosen samples from the food experiment with different barcodes. Ideally, we would have liked to see:

a/ a similar number of OTU's between the three technical replicas of the same sample. This was the case (see column Total # OTUs in Table 1).

b/ a large proportion (if not all) of OTU's to be shared between technical replicates. Only a small fraction (generally < 23%) of the OTUs appeared in only one replicate, except for replica Pm3Ea, where 40% of the OTUs were uniquely found in that replica. Most OTUs were thus shared between at least two replicates (Fig. 1).

c/ if unique OTUs are found, they should be there in very low frequency so that they will not have strong impacts on the final dataset. This was the case: the relative frequencies observed for each of the unique OTUs ranged between 0.01 and 0.21% confirming that they received only a very small fraction of the sequence reads (Table 2).

d/ the variation between technical replicates to be lower compared to the variation between biological replicates. We calculated Generalized Unifrac distances between the technical and biological replicates and found indeed that the variation among the technical replicates was lower than that compared to the biological replicates (Fig. 2).

Table 1: Total number of reads and OTUs for each of the three technical replicates for treatment Pm1B, Pm3E and Pm3bact. Replicates are indicated with letters a, b and c. "Unique OTUs" are OTUs that were only found in that particular replica. "OTUs_Shared_in_2_Repl" are OTUs that were shared between two replicates of the treatment; "OTUs_Shared_in_3_Repl" are OTUs that were present in all three replicates of the treatment.

| | Total # reads | Total # OTUs | Unique OTUs | OTUs_Shared_in_2_Repl | OTUs_Shared_in_3 Repl |
|---|---|---|---|---|---|
| Pm1Ba | 52894 | 581 | 87 | 222 | 272 |
| Pm1Bb | 56379 | 609 | 99 | 238 | 272 |
| Pm1Bc | 68788 | 651 | 137 | 242 | 272 |
| Pm3Ea | 136469 | 697 | 280 | 247 | 170 |
| Pm3Eb | 107838 | 497 | 66 | 261 | 170 |
| Pm3Ec | 125507 | 436 | 102 | 164 | 170 |
| Pm3bacta | 42930 | 419 | 81 | 173 | 165 |
| Pm3bactb | 68610 | 455 | 77 | 213 | 165 |
| Pm3bactc | 44634 | 323 | 28 | 130 | 165 |

Table 2: Calculation of the maximum relative frequency of unique OTUs per replica. For each replica, the frequency of the most abundant OTU is calculated ("Max_sequence count") and divided by the total number of sequence reads obtained for that replica ('Total reads') to yield the relative frequency in each OTU.

| | PM3bactc1a | PM3bactc1b | PM3bactc1c | PM1B1b | PM1B1a | PM1B1c | PM3E10a | PM3E10c | PM3E10b |
|---|---|---|---|---|---|---|---|---|---|
| Max_sequence count | 70 | 7 | 4 | 110 | 76 | 22 | 271 | 33 | 73 |
| Total reads | 41982 | 59285 | 42921 | 51587 | 54788 | 66762 | 133859 | 106476 | 123950 |
| Relative frequency | 0.17 | 0.01 | 0.01 | 0.21 | 0.14 | 0.03 | 0.20 | 0.03 | 0.06 |

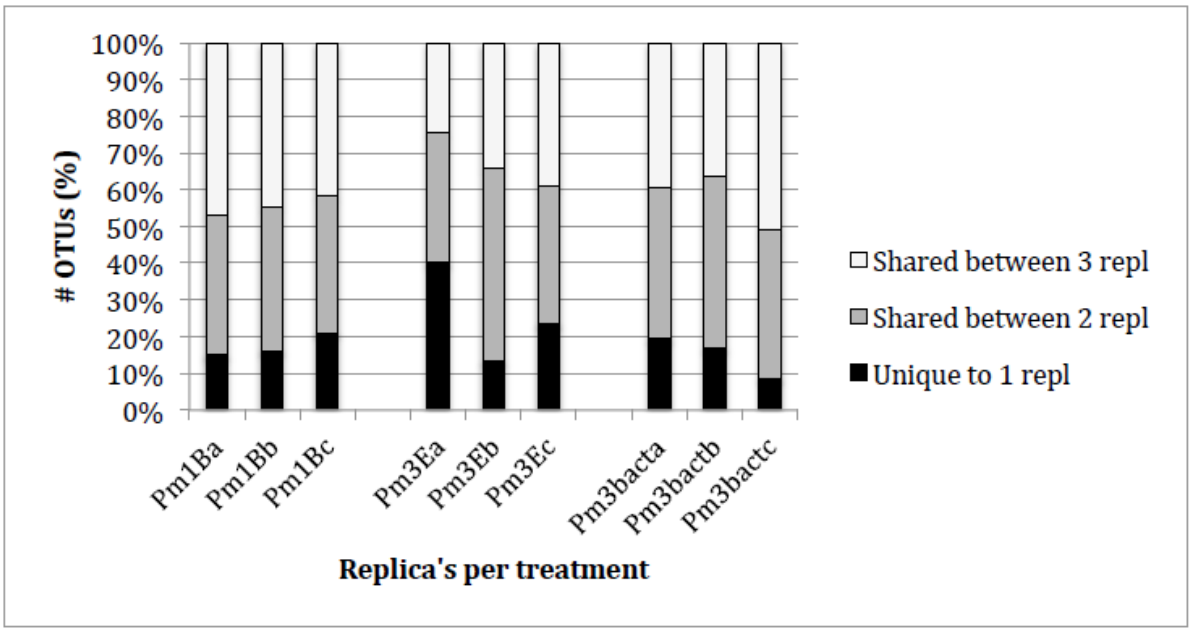



Figure 1: Percentage of OTUs uniquely found in each replica, shared between two replicas and shared between three replicas.

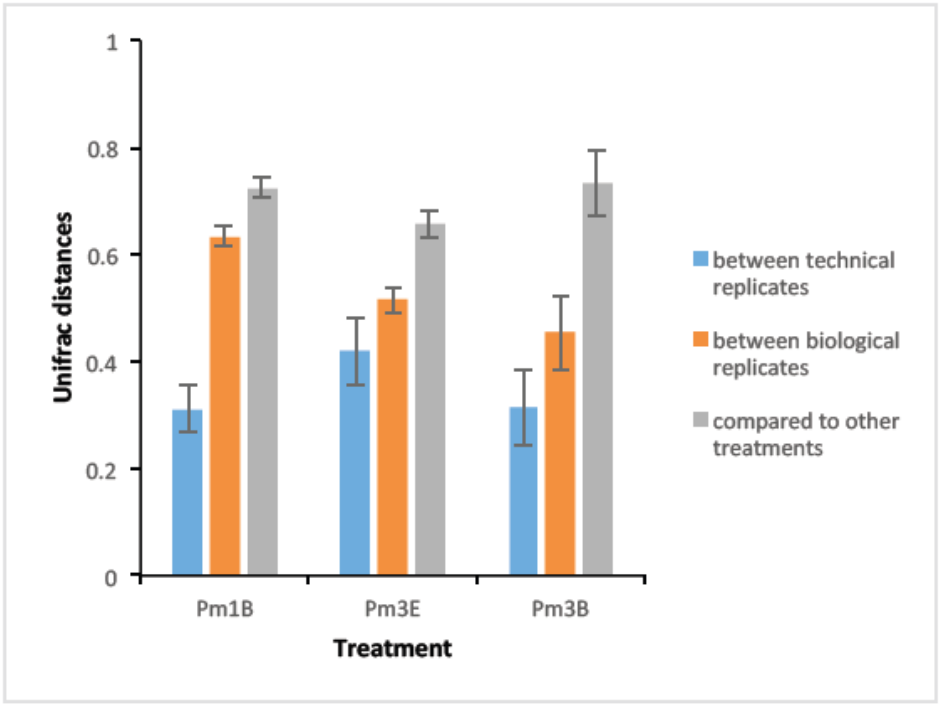



Figure 2: Average Unifrac distances between technical replicates and biological replicates of each of three treatments (Pm1B, Pm3E and Pm3B).

**Appendix S5**: Figures related to alpha diversity and beta diversity measurements of the forward dataset generated using the 454 platform of the field specimens.

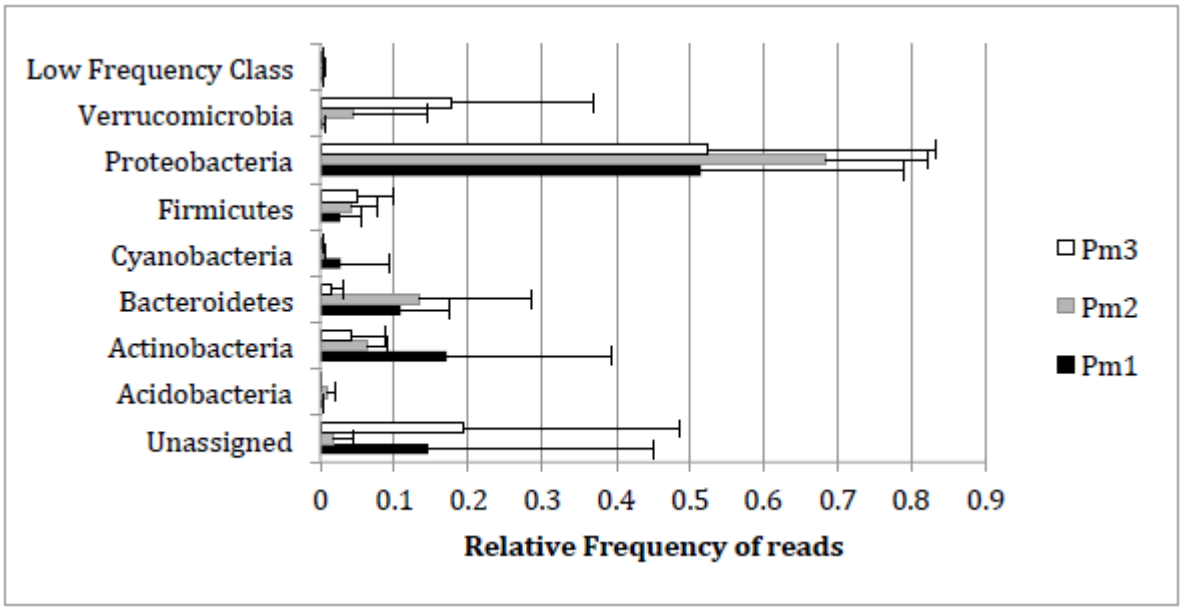



Figure 1: Taxonomic assignments at the phylum level of representative sequences of each OTU from the Forward dataset. Relative frequency of reads below 0.025 are pooled in a 'Low frequency Class'. Bars represent average values of six specimens for species Pm1 (black), Pm2 (grey) and Pm3 (white). Error bars are standard deviations based on six replicates from each species.

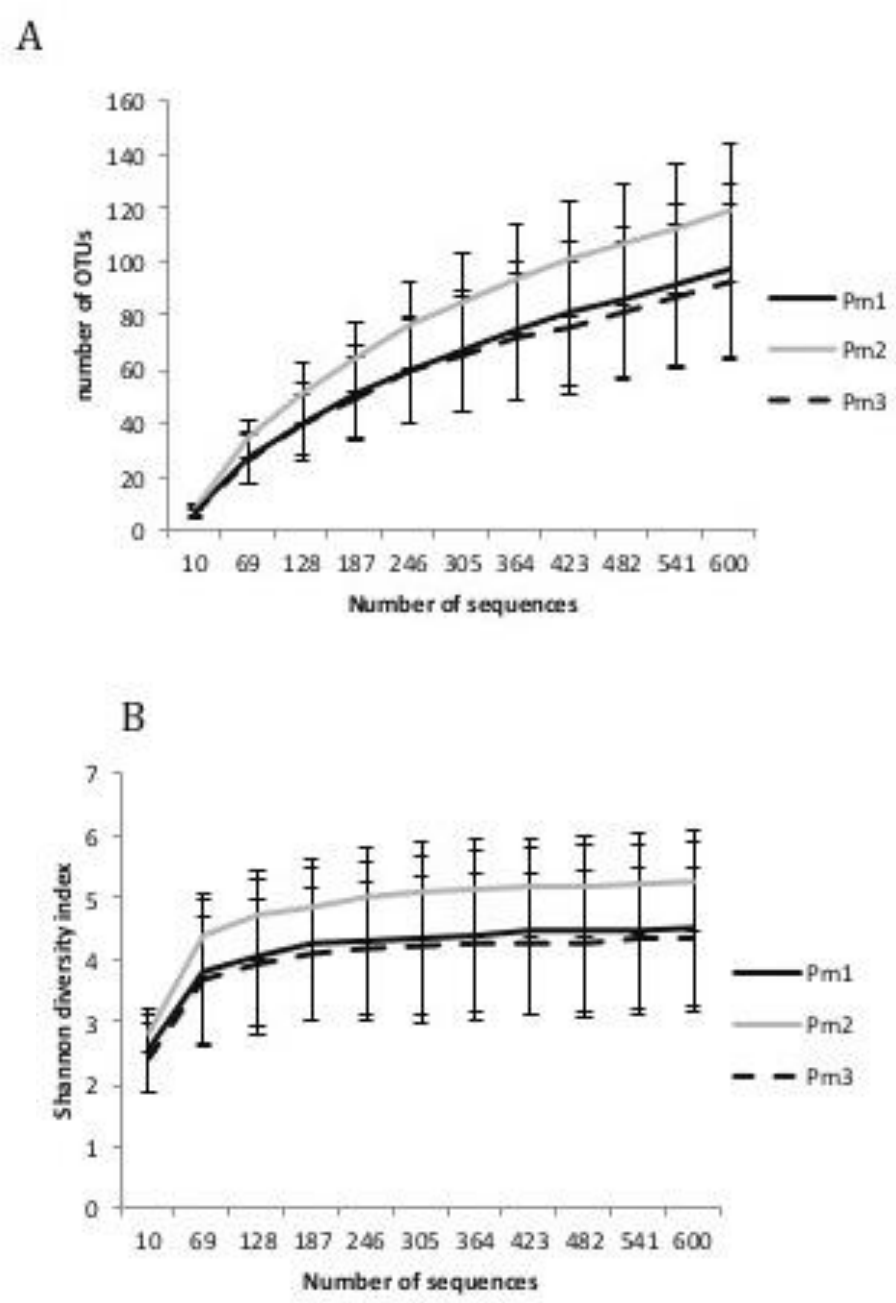



Figure 2: Rarefaction curves of the number of observed OTUs (A) and Shannon index (B) for each species based on Forward dataset. Error bars were calculated from the variance of the respective parameter drawn in 10 randomizations at each sample size.

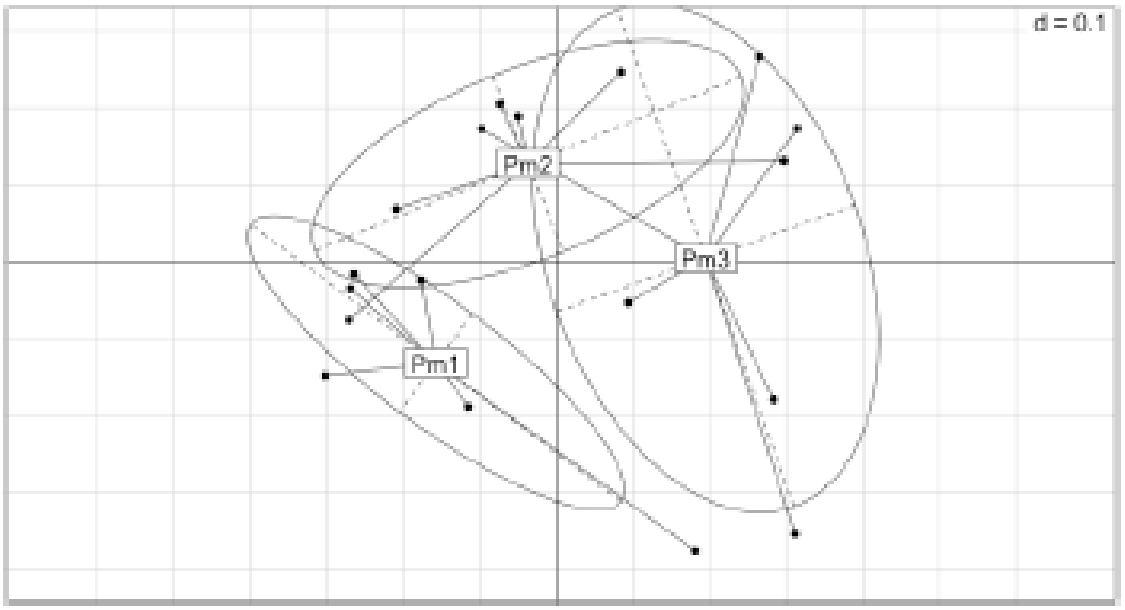
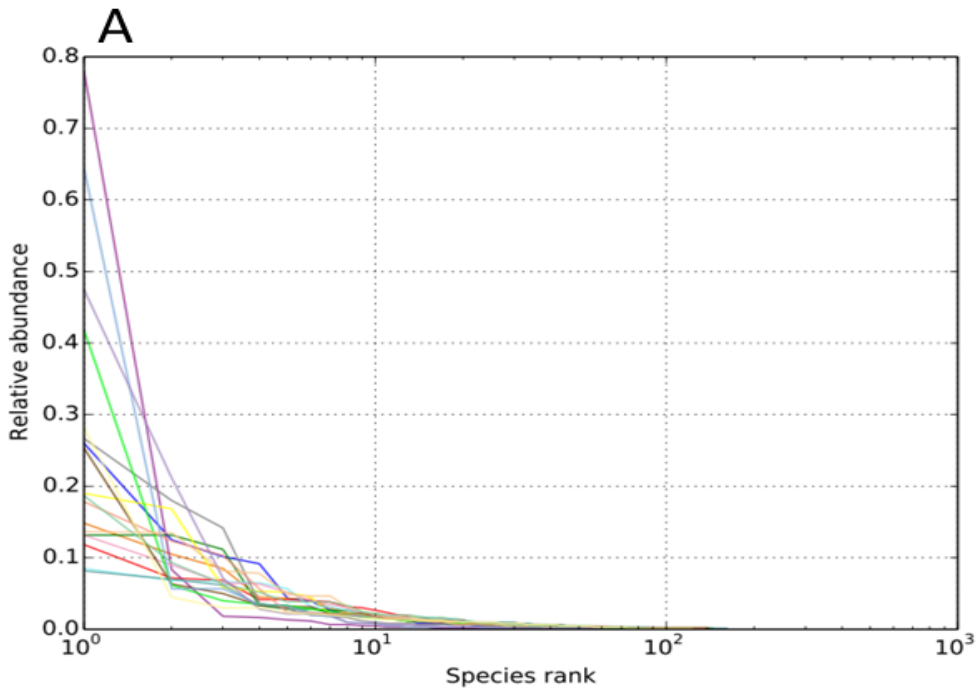
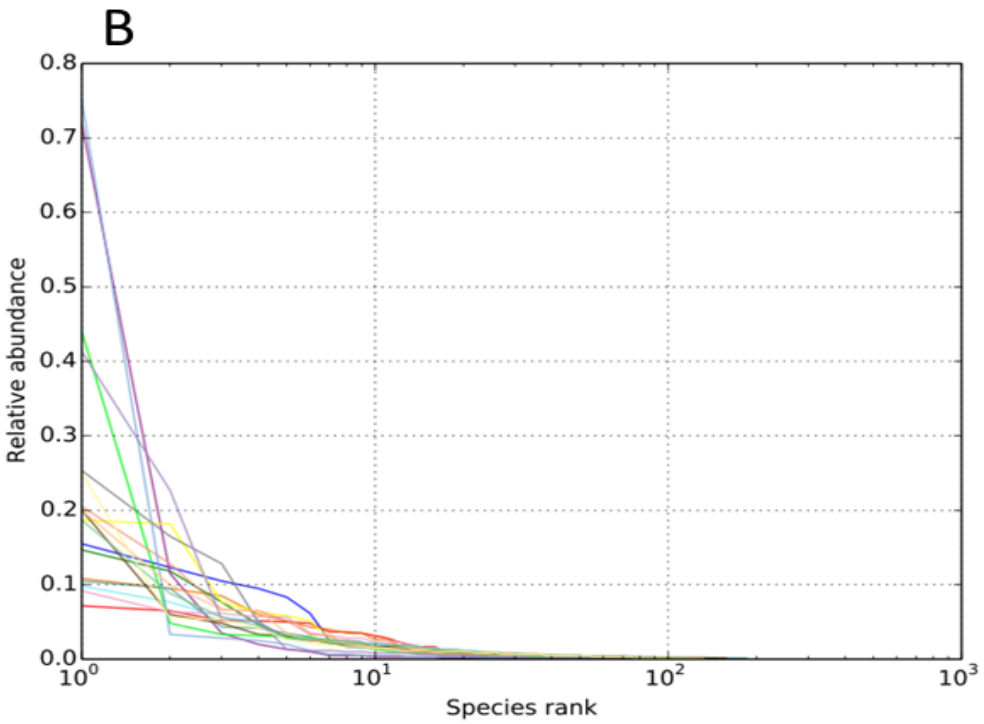



Figure 3: Principal coordinates analysis plot based on Generalized Unifrac distances between 18 nematode specimens after rarefaction of the forward dataset at 600 sequences per specimen. Intraspecific distances for Pm1, Pm2 and Pm3 are encircled.

**Appendix S6:** Rank abundance plots of OTUs from each of the 18 field specimens. Reads were generated with the 454 platform. Note the log scale of the X-axis. A/ Forward dataset; B/ Reverse dataset. Each line corresponds to one specimen.



**Appendix S7:** Taxonomic assignment of the core OTUs. Forward 97%: OTUs generated using the Forward dataset with OTU clustering at 97%; Reverse 97%: OTUs generated based on the Reverse dataset with OTU. OTUs that are present in all six specimens of species Pm1, Pm2 and Pm3 are indicated by "x". Core OTUs that are present in at least one specimen of the other species are indicated by "I".

| OTU ID | Pm1 | Pm2 | Pm3 | Taxonomy |
|---|---|---|---|---|
| Forward 97% | | | | |
| 209124 | x | x | I | Bacteroidetes, Flavobacteriia, Flavobacteriales, Weeksellaceae, Cloacibacterium |
| 4330856 | x | x | I | Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, |
| 741010 | x | I | I | Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio |
| New.ReferenceOTU34 | x | I | I | Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio |
| 4334053 | x | x | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, |
| 699789 | | I | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| 4449456 | I | I | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| 284413 | I | x | I | Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae, |
| 154604 | I | x | I | Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae, |
| Reverse 97% | | | | |
| **200979** | I | x | I | Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae, Microbacterium |
| 263590 | I | x | I | Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae, Microbacterium |
| 4437011 | I | x | I | Bacteroidetes, Flavobacteriia, Flavobacteriales, Weeksellaceae, Chryseobacterium |
| **310003** | | I | x | Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae |
| 4330856 | x | x | I | Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, |
| 4295954 | I | I | x | Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Acidovoraxcaeni |
| 4455981 | x | I | I | Proteobacteria, Deltaproteobacteria, Bdellovibrionales, Bacteriovoracaceae, |
| 741010 | x | I | I | Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio |
| 2967255 | x | I | I | Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio |
| **4334053** | x | x | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae |
| 720489 | | | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| **4449456** | | I | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| 4449458 | I | x | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |

| Reverse 99% | | | | |
|---|---|---|---|---|
| New.CleanUp.ReferenceOTU8140 | I | x | I | Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae, Microbacterium |
| 1102856 | x | I | I | Actinobacteria, Actinobacteria, Actinomycetales, Propionibacteriaceae, Propionibacterium, acnes |
| 1038849 | x | x | I | Bacteroidetes, Flavobacteriia, Flavobacteriales, Weeksellaceae, Chryseobacterium |
| 4330856 | x | x | I | Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae |
| 822041 | | I | x | Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae |
| 1061429 | x | x | I | Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae |
| New.CleanUp.ReferenceOTU11073 | x | I | I | Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio |
| 1025018 | x | x | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae |
| 1089344 | x | x | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae |
| 972803 | I | I | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| 960682 | | | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| New.CleanUp.ReferenceOTU6513 | | | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| New.CleanUp.ReferenceOTU8300 | I | I | x | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter |
| 1105959 | x | I | I | Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Enhydrobacter |

**Appendix S8:** Summary of the sequence data from the MiSeq run on the specimens of the food experiment. Summary of the sequence data from a separate MiSeq run containing three biological replicas of the *E. coli* suspension are also provided.

| Food experiment | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage | Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage |
| Pm1B1a | 54180 | 52894 | 51587 | 710 | 0.99 | Pm1E1 | 397754 | 391753 | 198450 | 2418 | 0,99 |
| Pm1B1b | 57938 | 56379 | 54788 | 789 | 0.99 | Pm1E2 | 79175 | 78438 | 77121 | 1150 | 0,97 |
| Pm1B1c | 71544 | 68788 | 66762 | 777 | 0.99 | Pm1E3 | 449025 | 443384 | 433624 | 2154 | 0,99 |
| Pm1B1 | 183662 | 178061 | 173137 | 1536 | 0.99 | Pm1E4 | 246629 | 243122 | 238173 | 1596 | 0,99 |
| Pm1B2 | 258860 | 257274 | 253159 | 2048 | 0.99 | Pm1E5 | 997926 | 989814 | 972963 | 3541 | 0,99 |
| Pm1B3 | 322913 | 303985 | 303985 | 6875 | 0.97 | Pm1E6 | 308058 | 306201 | 302576 | 1568 | 0,99 |
| Pm1B4 | 251187 | 249546 | 245673 | 1640 | 0.99 | Pm1E7 | 574878 | 572322 | 565391 | 1463 | 0,99 |
| Pm1B5 | 782833 | 775715 | 762658 | 4060 | 0.99 | Pm1E8 | 203608 | 202362 | 200038 | 1118 | 0,99 |
| Pm1B6 | 784471 | 776525 | 761842 | 3369 | 0.99 | Pm1E9 | 265059 | 263801 | 260447 | 1760 | 0,99 |
| Pm1B7 | 340165 | 335639 | 329048 | 2337 | 0.99 | Pm1E10 | 389187 | 387422 | 383213 | 1817 | 0,99 |
| Pm1B8 | 160028 | 158696 | 155969 | 1802 | 0.99 | | | | | | |
| Pm1B9 | 364867 | 362186 | 362186 | 7864 | 0.97 | | | | | | |
| Pm1B10 | 122397 | 121013 | 118399 | 1697 | 0.99 | | | | | | |
| total | 3571383 | 3518640 | 3466056 | 21774 | | | 3911299 | 3878619 | 3631996 | 10584 | |
| average | | | | 3322.8 | | | | | | 1858.5 | |

| Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage | Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pm3B1 | 481421 | 478578 | 470440 | 2871 | 0,99 | Pm3E1 | 570058 | 565998 | 557309 | 2603 | 0,99 |
| Pm3B2 | 505853 | 444182 | 437742 | 6875 | 0,97 | Pm3E2 | 369151 | 362186 | 362186 | 7864 | 0,98 |
| Pm3B3 | 418275 | 383985 | 303985 | 1979 | 0,99 | Pm3E3 | 262063 | 257821 | 252830 | 1982 | 0,99 |
| Pm3B4 | 460394 | 416041 | 410721 | 2632 | 0,99 | Pm3E4 | 439684 | 434603 | 426370 | 3410 | 0,99 |
| Pm3B5 | 511810 | 456470 | 449560 | 1546 | 0,99 | Pm3E5 | 352990 | 349212 | 343401 | 2238 | 0,99 |
| Pm3B6 | 399667 | 389922 | 306457 | 2540 | 0,99 | Pm3E6 | 277667 | 275888 | 271753 | 1990 | 0,99 |
| Pm3B7 | 532983 | 336437 | 329652 | 3324 | 0,99 | Pm3E7 | 377883 | 375053 | 369177 | 2049 | 0,99 |
| Pm3B8 | 532823 | 528457 | 517876 | 1517 | 0,99 | Pm3E8 | 496613 | 493721 | 487073 | 1857 | 0,99 |
| Pm3B9 | 445151 | 231459 | 228153 | 2271 | 0,99 | Pm3E9 | 336438 | 333999 | 328833 | 2310 | 0,99 |
| Pm3B10 | 446659 | 442031 | 433946 | 2595 | 0,99 | Pm3E10 | 372684 | 369814 | 364285 | 2277 | 0,99 |
| | | | | | | Pm3E10a | 137776 | 136469 | 133859 | 1293 | 0,99 |
| | | | | | | Pm3E10b | 108618 | 107838 | 106476 | 903 | 0,99 |
| | | | | | | Pm3E10c | 126290 | 125507 | 123950 | 849 | 0,99 |
| total | 4735036 | 4107562 | 3888532 | 15621 | | | 3855231 | 3818295 | 3763217 | 16963 | |
| average | | | | 2815 | | | | | | 2858 | |

| Stock and bacterial mix | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage | Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage | Samples | raw sequences | sequences after denoising | After open reference picking | #OTU_>1 | Good's coverage |
| Pm1C1 | 203843 | 202833 | 198450 | 1873 | 0.99 | Pm3C1a | 43253 | 42930 | 41982 | 597 | 0.99 | bactmixa | 278123 | 276469 | 270737 | 2197 | 0,98 |
| Pm1C2 | 272281 | 271027 | 266233 | 2120 | 0.99 | Pm3C1b | 69039 | 68610 | 59285 | 601 | 0.99 | bactmixb | 379113 | 375540 | 367033 | 2795 | 0,98 |
| | | | | | | Pm3C1c | 44938 | 44634 | 42921 | 397 | 1.00 | | | | | | |
| | | | | | | Pm3C1 | 157230 | 156174 | 144188 | 1053 | 0.99 | | | | | | |
| | | | | | | Pm3C2 | 248463 | 246977 | 242761 | 1550 | 0.99 | | | | | | |
| | 476124 | 473860 | 464683 | 2767 | | | 405693 | 403151 | 386949 | 1903 | | | 657236 | 652009 | 637770 | 5651 | |

| *Escherichia coli* suspension (sequenced on a separate Miseq run as part of a follow-up experiment) | | | | | |
|---|---|---|---|---|---|
| Samples | raw sequences | sequences after filtering | After open reference picking | #OTU_>1 | Good's coverage |
| EcoliA | 22909 | 22149 | 21306 | 573 | 0.99 |
| EcoliB | 27226 | 26493 | 25648 | 538 | 0.99 |
| EcoliC | 28914 | 28159 | 27300 | 546 | 0.99 |

**Appendix S9:** Taxonomic composition of the bacterial communities at the phylum, class and family level of the food experiment based on MiSeq sequencing of the 16S rRNA gene

The microbiomes of all samples were clearly dominated by Proteobacteria and Bacteroidetes (Fig. 1). Proteobacteria was the most dominant phylum in both food treatments of Pm1 (63% for B treatment and 72% for E treatment) and Pm3 (55% for B treatment and 37% for E treatment). Bacteriodetes was the second most dominant phylum for both species (>7.5% in all four treatments). The Actinobacteria were also present in all specimens, but reached much lower abundances than the Proteobacteria and Bacteroidetes. The Planctomycetes, Firmicutes and Cyanobacteria had low frequencies and were found in 95%, 95% and 93 % of all the samples, respectively. The samples in which these phyla were absent belonged to the *E.coli* treatment. A high frequency of unassigned OTUs was prominent in all specimens of Pm3 (4.8% and 6.3% for Pm1B and Pm1E, respectively and 55.1% and 37.3% for Pm3B and Pm3E, respectively).

Within the Proteobacteria, the Alpha and Gammaproteobacteria reached comparable ratios in the microbiomes of the four food treatments. For Pm1B, almost all specimens were dominated by Alphaproteobacteria, while half of the specimens of the *E.coli* treatment were dominated by Alphaproteobacteria and the other half by Gammaproteobacteria (Fig. 2a). For Pm3, the Gammaproteobacteria dominated in almost all specimens, regardless the food that had been offered (Fig. 2b). Within the Alphaproteobacteria 10 orders and 35 families were found. Both food treatments of Pm1 and Pm3 were dominated by the Rhizobiales (with high abundances of the Rhizobiaceae (Fig. 3)). The family of the Rhodobacteraceae was abundant in all Pm1 specimens, but for Pm3 this family was more abundant in the specimens that received *E. coli* as food source. The Rhodobacteraceae was also the most abundant family in the stock cultures of both species (Fig. 3). Within the Gammaproteobacteria 18 orders and 54 families were found. Vibrionales was the most abundant order for both stock cultures, the *E.coli* treatment of Pm1 and Pm3 and the bacterial mixture treatment of Pm1 and consisted almost exclusively of Pseudoalteromonadaceae and Vibrionaceae (Fig. 4). Pm1 and Pm3 worms that had been fed the bacterial mixture further contained a higher amount of Alteromonadaceae than those worms that had been fed *E. coli*. Importantly, the composition at the family level was appears to be different between worms that had been fed the bacterial mixture and those that had been fed *E. coli*, and the composition of the bacterial mix was similar to the composition of the worms that had been fed this mixture (Fig. 4). The Beta, Delta and Epsilon Proteobacteria contained only 9, 11 and 1 order, respectively. Within the

Bacteriodetes 7 orders and 26 families were observed. Flavobacteriales was the most abundant order (more than 60%) for both food treatments and the stock culture of Pm1 and Pm3. Flavobacteriaceae was the most abundant family within this order for all these treatments, and was more abundant in the Pm1 specimens than in the Pm3 specimens. In contrast, the order Saprospirales (with almost exclusively the family Saprospiraceae) was prominent in almost all samples, but reached higher abundances in all Pm3 specimens than in the Pm1 specimens. It was also the dominant family in the pure bacterial mixture (Fig. 5). For the bacterial mixture treatment of Pm1, the Cryomorphaceae was also present in a high abundance in several specimens (Fig. 5), while this family was almost absent in the other treatment and species.

The Actinobacteria consisted of 7 orders and 47 families with the order Actinomycetales comprising more than 95%. Within this order 33 families occurred with Microbacteriaceae being the most dominant in all the treatments (at least 70%) (Fig. 6).

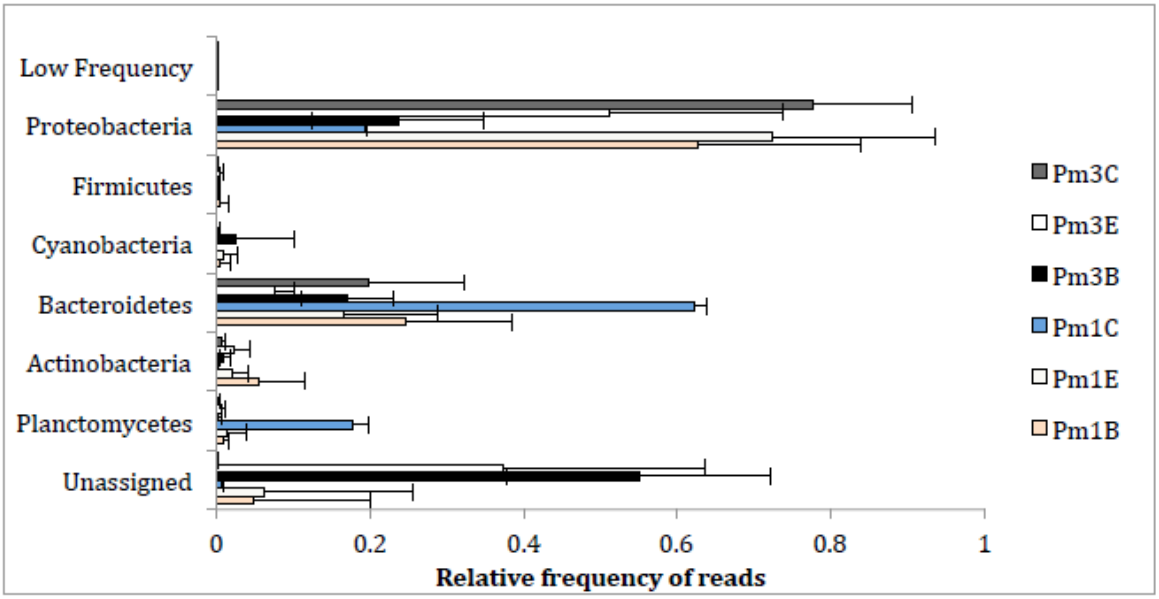
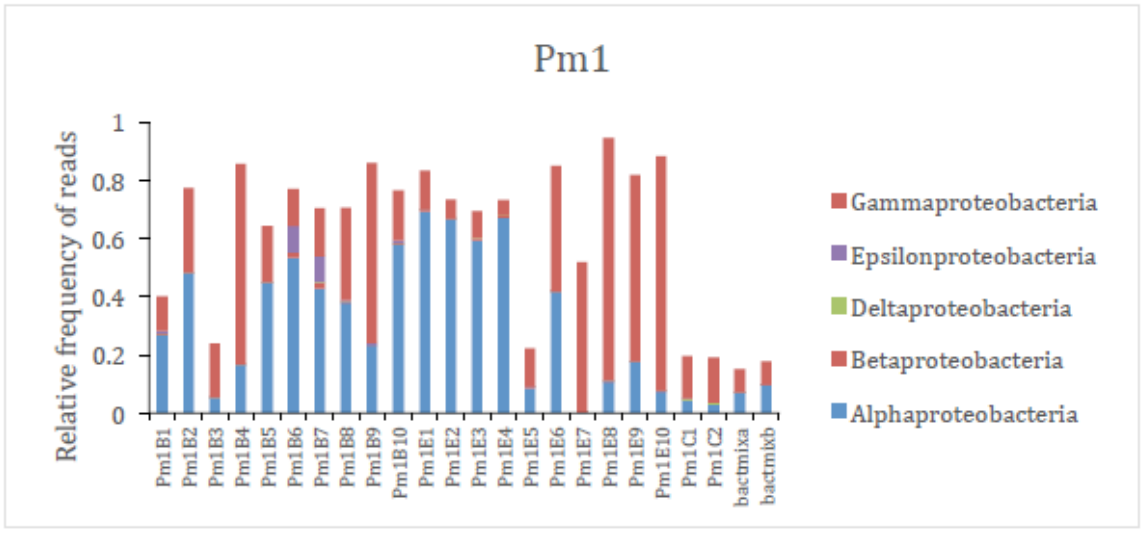



Figure 1: Taxonomic assignments of reads at the phylum level with a relative frequency ≥0.025 for the four treatments of the food experiment, and for the two stock cultures. Taxa with frequencies below 0.025 are pooled in a 'Low frequency' class. Bars represent average values with standard deviations of ten specimens for each food treatment (Pm1B: Pm1 nematodes fed the bacterial mix; Pm 3B: Pm3 nematodes fed the bacterial mix; Pm1E: Pm1 nematodes fed *E.coli*; Pm3E: Pm3 nematodes fed *E. coli*) and two replicates from the agar of stock cultures of Pm1 (Pm1C) and Pm3 (Pm3C).



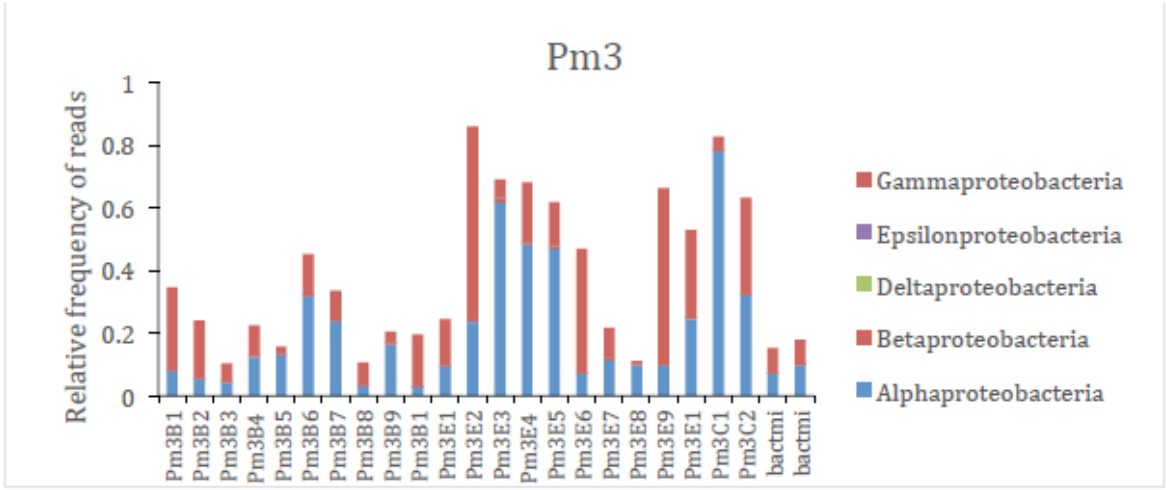
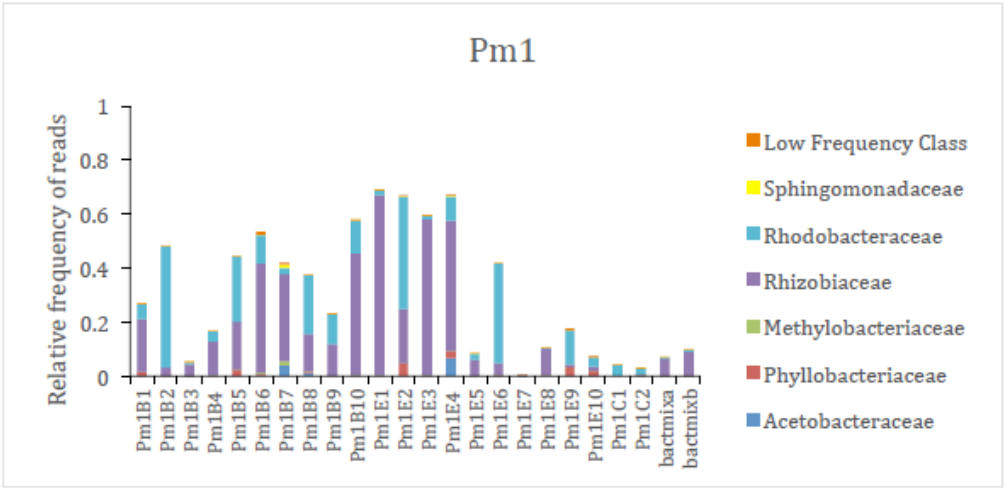




Figure 2: Taxonomic assignment of reads at the class level within Proteobacteria from the food experiment. A/ all 22 samples of Pm1. Pm1B1-10: 10 biological replicas of the bacterial mixture treatment; Pm1E1-10: 10 biological replicas of the *E. coli* treatment; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. B/ all 22 samples of Pm3. Sample codes as for Pm1.



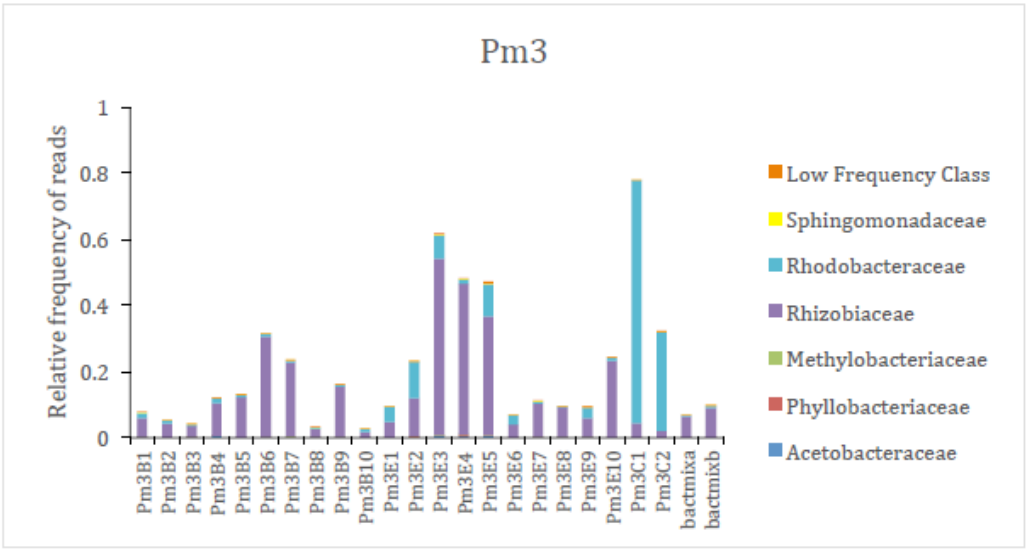




Figure 3: Taxonomic assignment of reads at the family level within Alphaproteobacteria for the food experiment. The six most abundant families are shown, the 29 remaining families are pooled in a "Low Frequency Class". A/ all 22 samples of Pm1. Pm1B1-10: 10 biological replicas of the bacterial mixture treatment; Pm1E1-10: 10 biological replicas of the *E. coli* mixture treatment; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. B/ all 22 samples of Pm3. Sample codes as for Pm1.

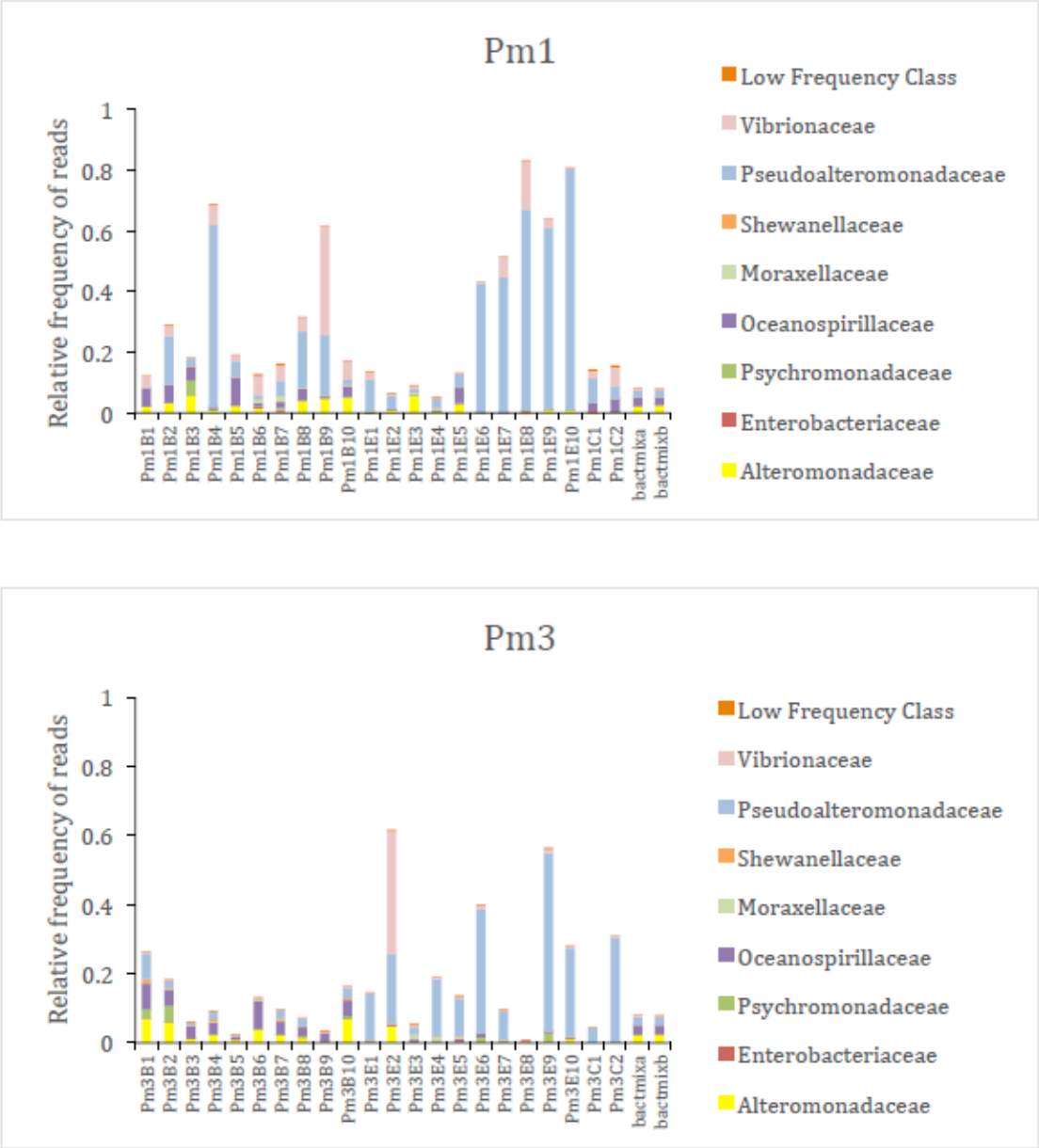



Figure 4: Taxonomic assignment of reads at the family level within Gammaproteobacteria for the food experiment. The eight most abundant families are shown, the 46 remaining taxa are pooled in a "Low Frequency Class ". A/ all samples of Pm1. Pm1B1-10: 10 biological replicas of the bacterial mixture treatment; Pm1E1-10: 10 biological replicas of the *E. coli* mixture treatment; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. B/ all 22 samples of Pm3. Sample codes as for Pm1.

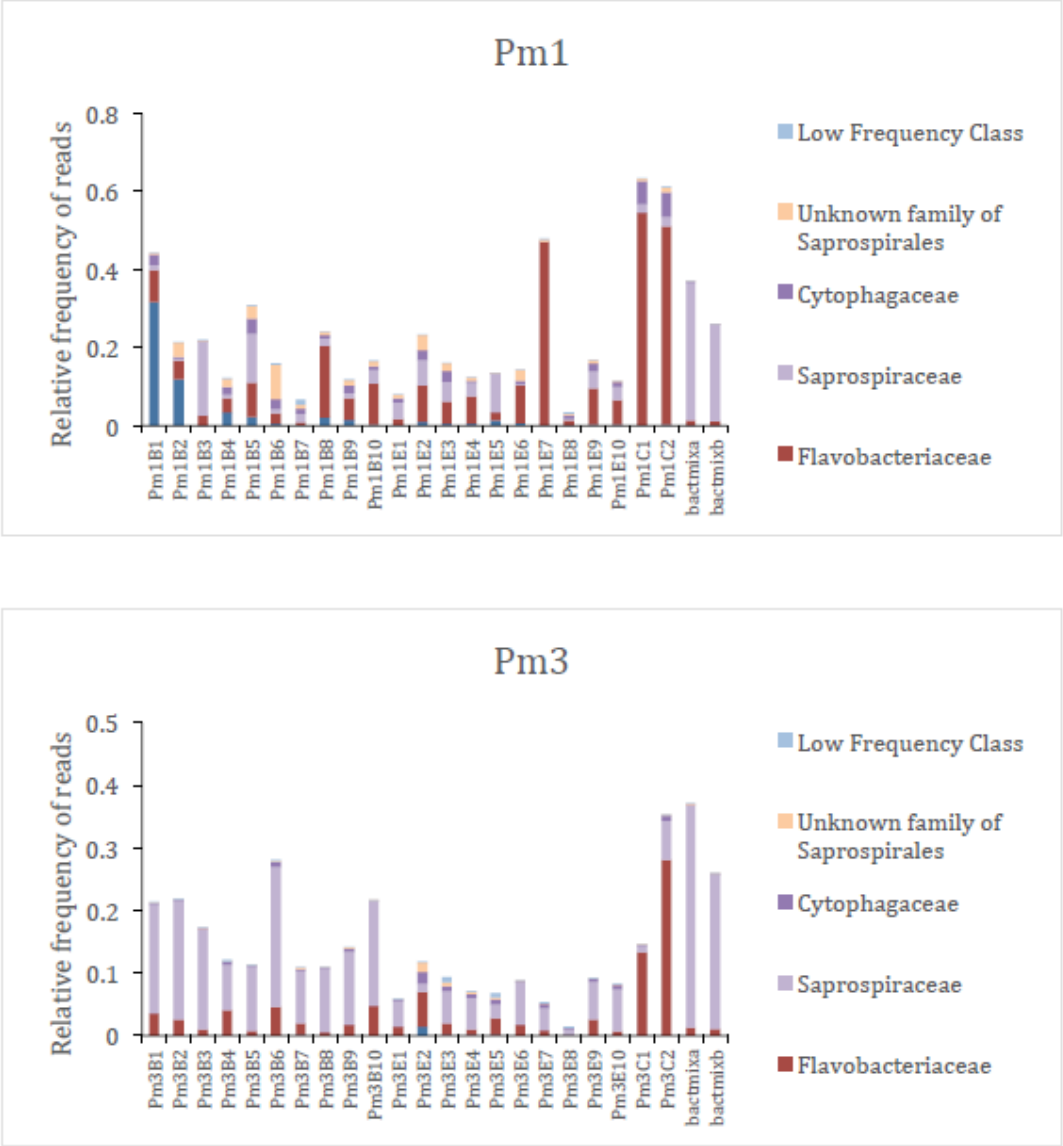



Figure 5: Taxonomic assignment of reads at the family level within Bacteroidetes for the food experiment. The five most abundant families are shown, the 21 remaining families are pooled in a "Low Frequency Class ". A/ all 22 samples of Pm1. Pm1B1-10: 10 biological replicas of the bacterial mixture treatment; Pm1E1-10: 10 biological replicas of the *E. coli* treatment; Pm1C1-2: two biological replicas of the agar from Pm1 cultures; bactmixa-b: two biological replicas of the bacterial mix. B/ all 22 samples of Pm3. Sample codes as for Pm1.

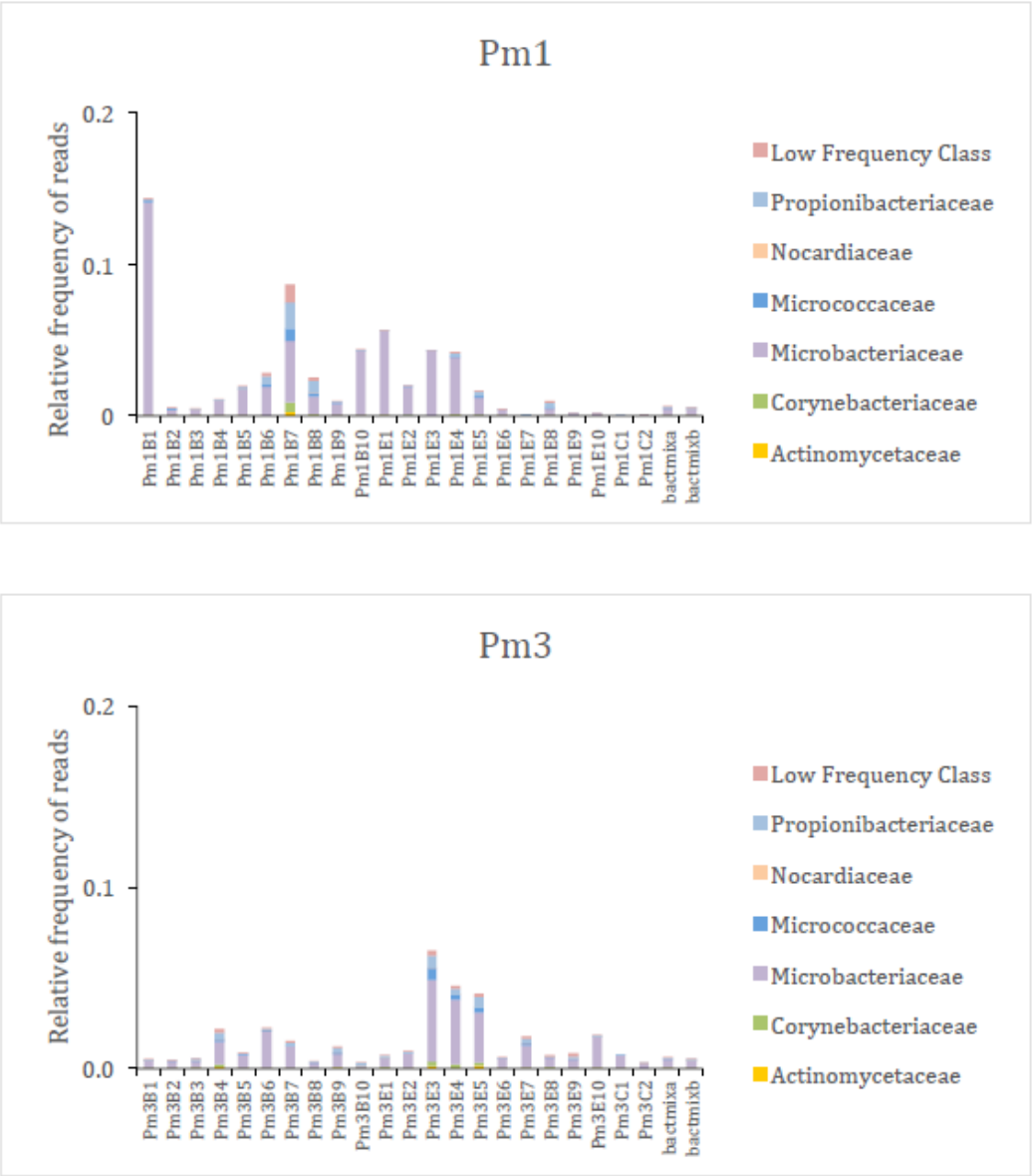



Figure 6: Taxonomic assignment of reads at the family level within Actinobacteria for the food experiment. The six most abundant families are shown, the 41 remaining families are pooled in a "Low Frequency Class ". A/ all 22 samples of Pm1. Pm1B1-10: 10 biological replicas of the bacterial mixture treatment; Pm1E1-10: 10 biological replicas of the *E. coli* treatment; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. B/ all 22 samples of Pm3. Sample codes as for Pm1.

**Appendix S10:** Graphs of alpha diversity (rarefaction curves of number of OTUs and Shannon Index, rank abundance plots) of the specimens of the food experiment.

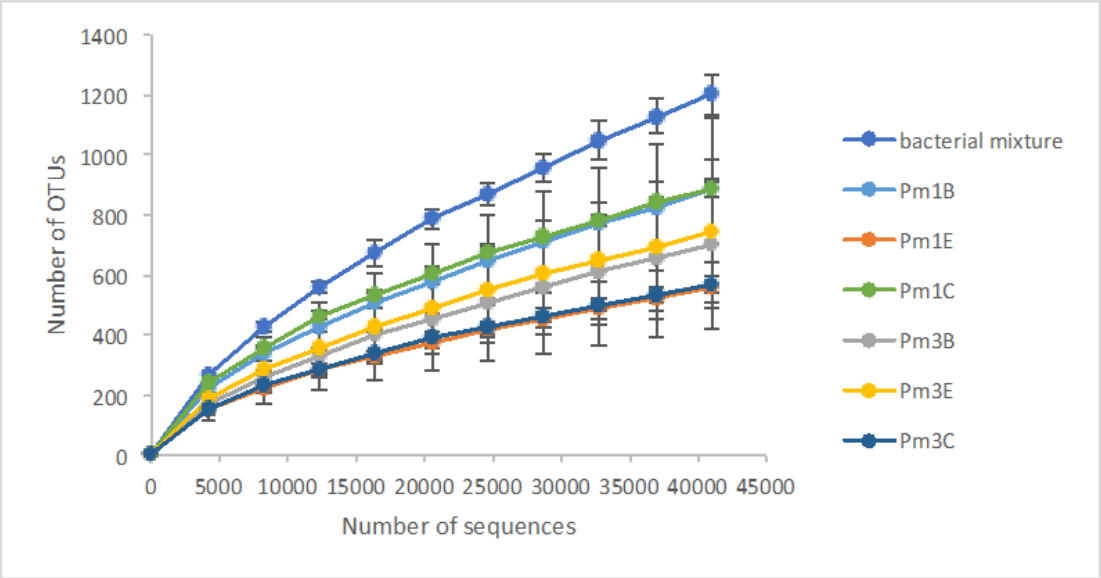



Figure 1: Rarefaction curves of the number of observed OTUs for each treatment of the food experiment (Pm1B, Pm1E, Pm3B, Pm3E), the agar from the stock cultures (Pm1C and Pm3C) and the pure bacterial mixture. Error bars were calculated from the variance of 10 randomizations at each sample size.

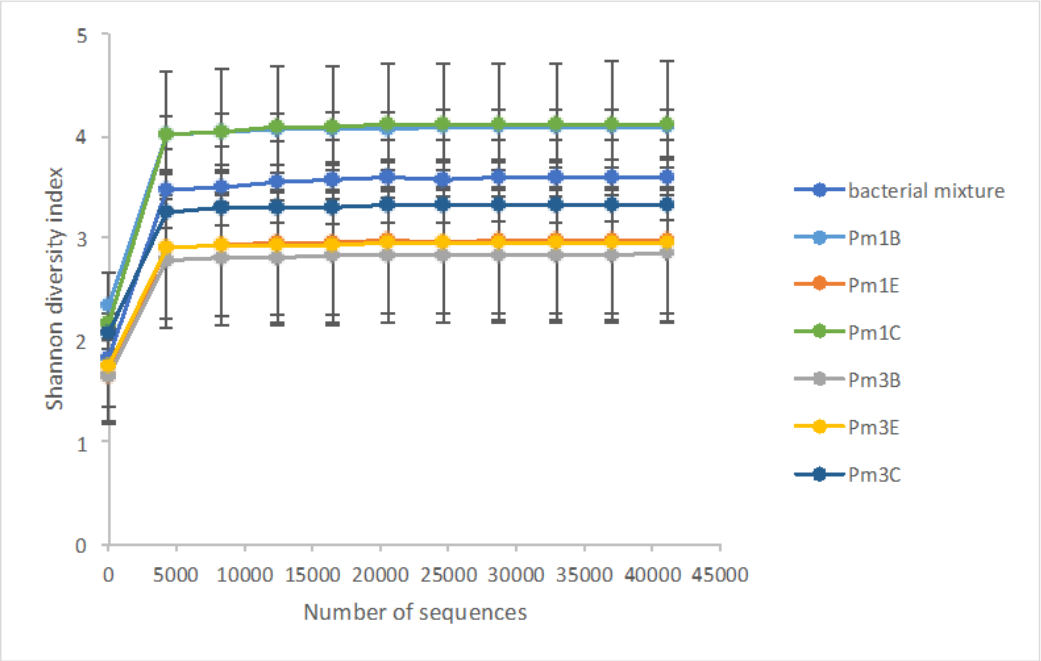
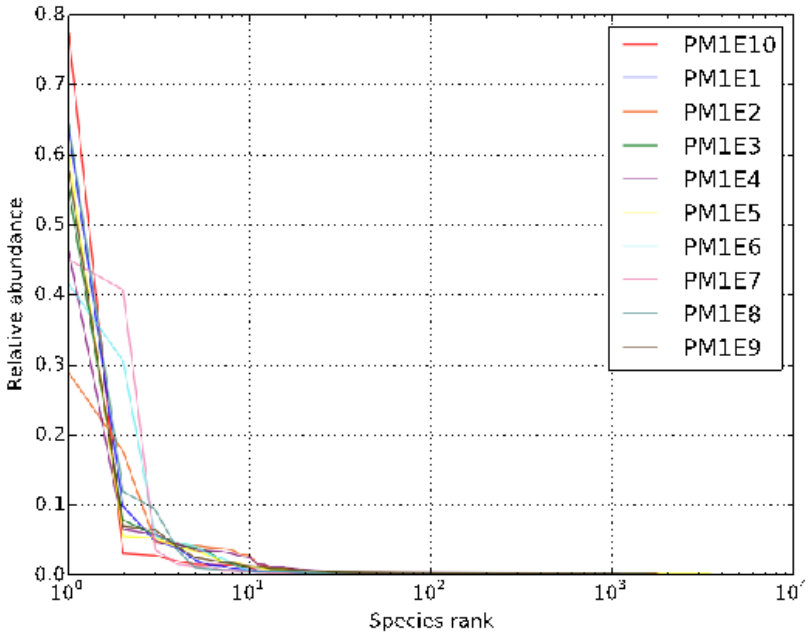



Figure 2: Rarefaction curves of the number of the Shannon Index for each treatment of the food experiment (Pm1B, Pm1E, Pm3B, Pm3E), the agar from the stock cultures (Pm1C and Pm3C) and the pure bacterial mixture. Error bars were calculated from the variance of 10 randomizations at each sample size.

A




B

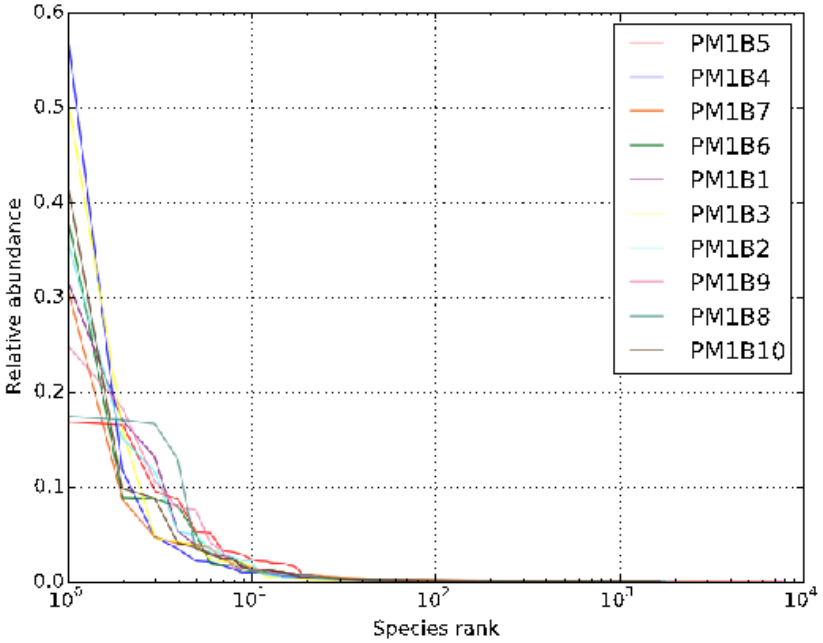
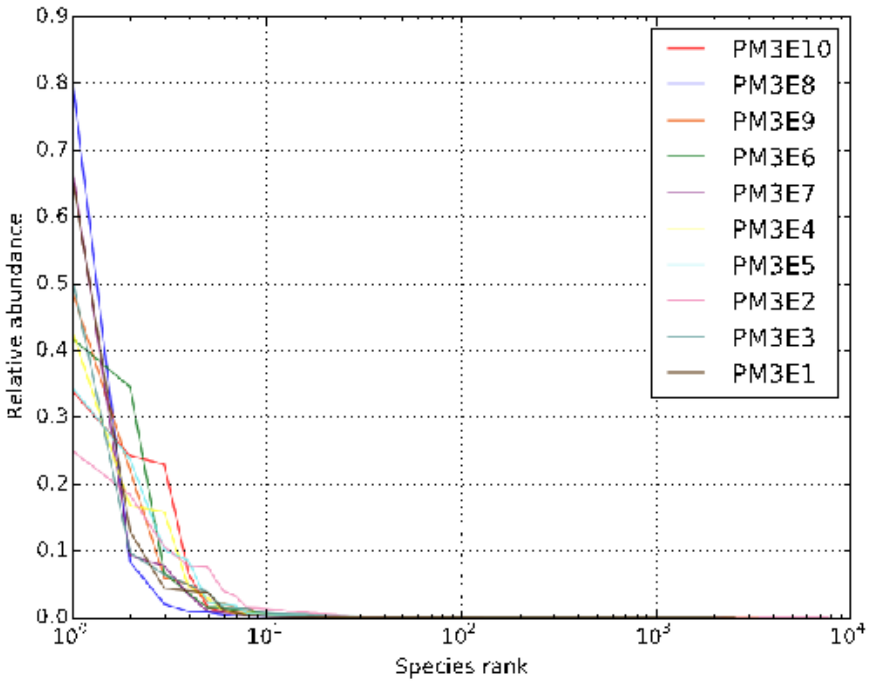



Figure 3: Rank abundance plots of OTUs from each of the 20 Pm1 specimens. Note the log scale of the X-axis. A/ *E. coli* fed worms; B/ Bacterial mix fed worms. Each line corresponds to one specimen.

A




B

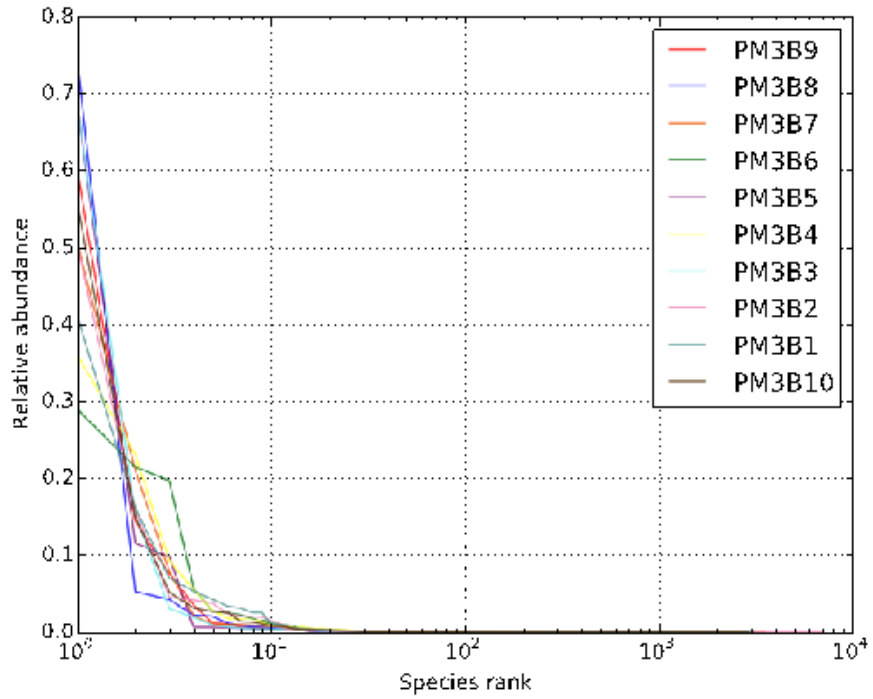



Figure 4: Rank abundance plots of OTUs from each of the 20 Pm3 specimens. Note the log scale of the X-axis. A/ *E. coli* fed worms; B/ Bacterial mix fed worms. Each line corresponds to one specimen.

**Appendix S11:** List of biomarker taxa identified by LeFSe for Pm1 and Pm3 from the food experiment. OTU ID and taxonomic assignment using Greengenes are included.

| Biomarker taxa for Pm1 | |
|---|---|
| OTU ID | Taxonomy |
| 84346 | Bacteroidetes |
| NewReferenceOTU2348 | Bacteroidetes |
| NewReferenceOTU2103 | Bacteroidetes |
| 394758 | Bacteroidetes |
| 783545 | Bacteroidetes |
| NewReferenceOTU590 | Bacteroidetes |
| NewReferenceOTU1082 | Bacteroidetes |
| 114234 | Bacteroidetes |
| 643716 | Bacteroidetes |
| 210280 | Bacteroidetes |
| 249383 | Bacteroidetes |
| 1032085 | Bacteroidetes |
| NewReferenceOTU4008 | Bacteroidetes |
| NewReferenceOTU1030 | Bacteroidetes |
| 4321726 | Bacteroidetes |
| NewReferenceOTU4188 | Bacteroidetes |
| 549386 | Bacteroidetes |
| NewReferenceOTU4168 | Bacteroidetes |
| 149448 | Bacteroidetes |
| NewReferenceOTU4195 | Bacteroidetes |
| NewReferenceOTU4168 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU4195 | Bacteroidetes.Flavobacteriia |
| 1032085 | Bacteroidetes.Flavobacteriia |
| 249383 | Bacteroidetes.Flavobacteriia |
| 4321726 | Bacteroidetes.Flavobacteriia |
| 84346 | Bacteroidetes.Flavobacteriia |
| 149448 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU1082 | Bacteroidetes.Flavobacteriia |
| 643716 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU590 | Bacteroidetes.Flavobacteriia |
| 783545 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU2348 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU2103 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU4008 | Bacteroidetes.Flavobacteriia |
| 394758 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU4188 | Bacteroidetes.Flavobacteriia |
| 114234 | Bacteroidetes.Flavobacteriia |

| | |
|---|---|
| NewReferenceOTU4168 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 394758 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU4008 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2103 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 4321726 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 114234 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 1032085 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 643716 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 149448 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU590 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 249383 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 84346 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU4195 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU1082 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2348 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 783545 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU4188 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2103 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae |
| NewReferenceOTU4008 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae |
| NewReferenceOTU4168 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae |
| NewReferenceOTU4188 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae |
| NewReferenceOTU4195 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae |
| NewReferenceOTU2103 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g |
| NewReferenceOTU4188 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g |
| NewReferenceOTU4195 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g |
| NewReferenceOTU4168 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g |
| NewReferenceOTU4195 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g.s |
| NewReferenceOTU4168 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g.s |
| NewReferenceOTU2103 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g.s |
| NewReferenceOTU4188 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Cryomorphaceae.g.s |
| 783545 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU1082 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 4321726 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 1032085 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU2348 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU590 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 394758 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 149448 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 249383 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 114234 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 84346 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 643716 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU2348 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g |
| 1032085 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g |

| | |
|---|---|
| 249383 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g |
| 1032085 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g.s |
| 249383 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g.s |
| NewReferenceOTU2348 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.g.s |
| 84346 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gMaribacter |
| 84346 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gMaribacter.s |
| 394758 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| NewReferenceOTU1082 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 114234 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 643716 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 783545 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 4321726 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| NewReferenceOTU590 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 149448 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella |
| 4321726 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 149448 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| NewReferenceOTU1082 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| NewReferenceOTU590 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 643716 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 783545 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 394758 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 114234 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gWinogradskyella.sthalassocola |
| 549386 | Bacteroidetes.Saprospirae |
| NewReferenceOTU1030 | Bacteroidetes.Saprospirae |
| 210280 | Bacteroidetes.Saprospirae |
| 210280 | Bacteroidetes.Saprospirae.Saprospirales |
| NewReferenceOTU1030 | Bacteroidetes.Saprospirae.Saprospirales |
| 549386 | Bacteroidetes.Saprospirae.Saprospirales |
| 210280 | Bacteroidetes.Saprospirae.Saprospirales. |
| 549386 | Bacteroidetes.Saprospirae.Saprospirales. |
| NewReferenceOTU1030 | Bacteroidetes.Saprospirae.Saprospirales. |
| 210280 | Bacteroidetes.Saprospirae.Saprospirales..g |
| 549386 | Bacteroidetes.Saprospirae.Saprospirales..g |
| NewReferenceOTU1030 | Bacteroidetes.Saprospirae.Saprospirales..g |
| 210280 | Bacteroidetes.Saprospirae.Saprospirales..g.s |
| 549386 | Bacteroidetes.Saprospirae.Saprospirales..g.s |
| NewReferenceOTU1030 | Bacteroidetes.Saprospirae.Saprospirales..g.s |
| NewReferenceOTU100 | Proteobacteria |
| 591923 | Proteobacteria |
| NewReferenceOTU2343 | Proteobacteria |
| NewReferenceOTU921 | Proteobacteria |
| NewReferenceOTU169 | Proteobacteria |
| NewReferenceOTU2218 | Proteobacteria |
| 593700 | Proteobacteria |

| | |
|---|---|
| 561165 | Proteobacteria |
| NewReferenceOTU1033 | Proteobacteria |
| NewCleanUpReferenceOTU150954 | Proteobacteria |
| 1108208 | Proteobacteria |
| 176420 | Proteobacteria |
| 750031 | Proteobacteria |
| NewReferenceOTU1334 | Proteobacteria |
| 251914 | Proteobacteria |
| 252007 | Proteobacteria |
| 542278 | Proteobacteria |
| 836362 | Proteobacteria |
| 309489 | Proteobacteria |
| NewReferenceOTU1521 | Proteobacteria |
| NewCleanUpReferenceOTU130132 | Proteobacteria |
| NewReferenceOTU3035 | Proteobacteria |
| NewReferenceOTU471 | Proteobacteria |
| NewReferenceOTU2011 | Proteobacteria |
| 158140 | Proteobacteria |
| 326373 | Proteobacteria |
| NewReferenceOTU408 | Proteobacteria |
| NewReferenceOTU3401 | Proteobacteria |
| 140787 | Proteobacteria |
| 808031 | Proteobacteria |
| 36441 | Proteobacteria |
| 837366 | Proteobacteria |
| 79817 | Proteobacteria |
| 19398 | Proteobacteria |
| NewCleanUpReferenceOTU220298 | Proteobacteria |
| NewCleanUpReferenceOTU117670 | Proteobacteria |
| NewCleanUpReferenceOTU92419 | Proteobacteria |
| 586746 | Proteobacteria |
| 84384 | Proteobacteria |
| 153173 | Proteobacteria |
| NewReferenceOTU1989 | Proteobacteria |
| 140920 | Proteobacteria |
| NewReferenceOTU419 | Proteobacteria |
| NewReferenceOTU2435 | Proteobacteria |
| 590614 | Proteobacteria |
| 4353625 | Proteobacteria |
| NewReferenceOTU2796 | Proteobacteria |
| 830290 | Proteobacteria |
| NewReferenceOTU3191 | Proteobacteria |
| 830696 | Proteobacteria |
| 518661 | Proteobacteria |

| | |
|---|---|
| NewReferenceOTU1187 | Proteobacteria |
| 140829 | Proteobacteria |
| 140860 | Proteobacteria |
| 562023 | Proteobacteria |
| 518661 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2011 | Proteobacteria.Alphaproteobacteria |
| 593700 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU1334 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU1187 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU1989 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU471 | Proteobacteria.Alphaproteobacteria |
| 590614 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU1033 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU3035 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU150954 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU220298 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU921 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU408 | Proteobacteria.Alphaproteobacteria |
| 750031 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU130132 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU419 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU169 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2218 | Proteobacteria.Alphaproteobacteria |
| 542278 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2343 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU100 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2435 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2796 | Proteobacteria.Alphaproteobacteria |
| 1108208 | Proteobacteria.Alphaproteobacteria |
| 326373 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU117670 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2435 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewCleanUpReferenceOTU220298 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewReferenceOTU3035 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewReferenceOTU2796 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| 518661 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewReferenceOTU1989 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewCleanUpReferenceOTU220298 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae |
| 518661 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae |
| NewReferenceOTU3035 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae |
| NewReferenceOTU2796 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae |
| NewReferenceOTU1989 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae |
| NewReferenceOTU2796 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g |
| NewCleanUpReferenceOTU220298 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g |

| | |
|---|---|
| NewReferenceOTU1989 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g |
| NewReferenceOTU3035 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g |
| NewReferenceOTU1989 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g.s |
| NewReferenceOTU2796 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g.s |
| NewReferenceOTU3035 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g.s |
| NewCleanUpReferenceOTU220298 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Phyllobacteriaceae.g.s |
| NewReferenceOTU2435 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae |
| NewReferenceOTU2435 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae.gAgrobacterium |
| NewReferenceOTU2435 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae.gAgrobacterium.s |
| NewReferenceOTU2343 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU100 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU1334 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewCleanUpReferenceOTU150954 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewCleanUpReferenceOTU117670 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU921 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU471 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 1108208 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewCleanUpReferenceOTU130132 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU2218 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU169 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU1033 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 326373 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU408 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 750031 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU1187 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 593700 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU2011 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 542278 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU419 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 590614 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU2218 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewCleanUpReferenceOTU117670 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU1033 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU419 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU100 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 1108208 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 326373 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU408 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU169 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 750031 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU2011 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 593700 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU471 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU921 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |

| | |
|---|---|
| NewReferenceOTU2343 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU1187 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewCleanUpReferenceOTU130132 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU1334 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewCleanUpReferenceOTU150954 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 590614 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 542278 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU2011 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU2218 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 1108208 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 593700 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU471 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU921 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU419 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewCleanUpReferenceOTU130132 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU408 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU1187 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU2343 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU1334 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 326373 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewCleanUpReferenceOTU150954 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 750031 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU100 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 542278 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewCleanUpReferenceOTU117670 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| 1108208 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU1334 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| 326373 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU2011 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU471 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| 542278 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU1187 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewCleanUpReferenceOTU130132 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| 593700 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU419 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU2343 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewCleanUpReferenceOTU117670 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU408 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU100 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| 750031 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU2218 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU921 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewCleanUpReferenceOTU150954 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU169 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gOctadecabacter |

| | |
|---|---|
| 590614 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gOctadecabacter |
| NewReferenceOTU169 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gOctadecabacter.s |
| 590614 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gOctadecabacter.s |
| 837366 | Proteobacteria.Gammaproteobacteria |
| 836362 | Proteobacteria.Gammaproteobacteria |
| 562023 | Proteobacteria.Gammaproteobacteria |
| 591923 | Proteobacteria.Gammaproteobacteria |
| 158140 | Proteobacteria.Gammaproteobacteria |
| 561165 | Proteobacteria.Gammaproteobacteria |
| 79817 | Proteobacteria.Gammaproteobacteria |
| 830290 | Proteobacteria.Gammaproteobacteria |
| 586746 | Proteobacteria.Gammaproteobacteria |
| 140787 | Proteobacteria.Gammaproteobacteria |
| 153173 | Proteobacteria.Gammaproteobacteria |
| 808031 | Proteobacteria.Gammaproteobacteria |
| NewCleanUpReferenceOTU92419 | Proteobacteria.Gammaproteobacteria |
| 309489 | Proteobacteria.Gammaproteobacteria |
| 36441 | Proteobacteria.Gammaproteobacteria |
| 4353625 | Proteobacteria.Gammaproteobacteria |
| 84384 | Proteobacteria.Gammaproteobacteria |
| 830696 | Proteobacteria.Gammaproteobacteria |
| 176420 | Proteobacteria.Gammaproteobacteria |
| 140920 | Proteobacteria.Gammaproteobacteria |
| NewReferenceOTU3191 | Proteobacteria.Gammaproteobacteria |
| 251914 | Proteobacteria.Gammaproteobacteria |
| NewReferenceOTU1521 | Proteobacteria.Gammaproteobacteria |
| 140829 | Proteobacteria.Gammaproteobacteria |
| 140860 | Proteobacteria.Gammaproteobacteria |
| 252007 | Proteobacteria.Gammaproteobacteria |
| 19398 | Proteobacteria.Gammaproteobacteria |
| NewReferenceOTU3401 | Proteobacteria.Gammaproteobacteria |
| 79817 | Proteobacteria.Gammaproteobacteria.Alteromonadales |
| 79817 | Proteobacteria.Gammaproteobacteria.Alteromonadales.Alteromonadaceae |
| 79817 | Proteobacteria.Gammaproteobacteria.Alteromonadales.Alteromonadaceae.g |
| 79817 | Proteobacteria.Gammaproteobacteria.Alteromonadales.Alteromonadaceae.g.s |
| 140860 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 586746 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 176420 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 830696 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 140787 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 19398 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 561165 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewCleanUpReferenceOTU92419 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 837366 | Proteobacteria.Gammaproteobacteria.Vibrionales |

| | |
|---|---|
| 309489 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 36441 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 836362 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU3191 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 140920 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 591923 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 808031 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 140829 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 158140 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 252007 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 251914 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 4353625 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 830290 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU3401 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU1521 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 153173 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 84384 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 562023 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 562023 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 808031 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 836362 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 561165 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 19398 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 140829 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 309489 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 84384 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 251914 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 140920 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 140860 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 4353625 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 140787 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 153173 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 591923 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| NewReferenceOTU3401 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 830696 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 158140 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 586746 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 36441 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 830290 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 4353625 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 36441 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 830696 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 830290 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 562023 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |

| | |
|---|---|
| 153173 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 836362 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 140920 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 561165 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 808031 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 140860 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 586746 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 158140 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 309489 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 251914 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 84384 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 140787 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 19398 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| NewReferenceOTU3401 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 140829 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 591923 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 808031 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 4353625 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 562023 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 140860 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 140787 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 19398 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 830290 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 561165 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 309489 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 153173 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 36441 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 140829 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 836362 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 830696 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 84384 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 251914 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 591923 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 158140 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| NewReferenceOTU3401 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 140920 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas.s |
| 4353625 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.gPseudoalteromonas |
| 837366 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| 252007 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| 176420 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewCleanUpReferenceOTU92419 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewReferenceOTU3191 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewReferenceOTU3191 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.g |
| NewReferenceOTU3191 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.g.s |

| Biomarkers for Pm3 | |
|---|---|
| OTU ID | Taxonomy |
| 278303 | Bacteroidetes |
| NewCleanUpReferenceOTU29602 | Bacteroidetes |
| 147086 | Bacteroidetes |
| NewReferenceOTU4131 | Bacteroidetes |
| NewReferenceOTU2357 | Bacteroidetes |
| NewReferenceOTU2438 | Bacteroidetes |
| NewReferenceOTU2455 | Bacteroidetes |
| NewCleanUpReferenceOTU155365 | Bacteroidetes |
| NewReferenceOTU2305 | Bacteroidetes |
| 4313779 | Bacteroidetes |
| NewReferenceOTU631 | Bacteroidetes |
| 147086 | Bacteroidetes.Flavobacteriia |
| NewCleanUpReferenceOTU29602 | Bacteroidetes.Flavobacteriia |
| 278303 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU2455 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU2305 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU2438 | Bacteroidetes.Flavobacteriia |
| NewReferenceOTU4131 | Bacteroidetes.Flavobacteriia |
| NewCleanUpReferenceOTU155365 | Bacteroidetes.Flavobacteriia |
| NewCleanUpReferenceOTU155365 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewCleanUpReferenceOTU29602 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 147086 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2438 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| 278303 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU4131 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2305 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewReferenceOTU2455 | Bacteroidetes.Flavobacteriia.Flavobacteriales |
| NewCleanUpReferenceOTU155365 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU4131 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 278303 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewCleanUpReferenceOTU29602 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU2438 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU2305 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| 147086 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewReferenceOTU2455 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae |
| NewCleanUpReferenceOTU29602 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| NewReferenceOTU2305 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| 147086 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| NewReferenceOTU4131 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| NewReferenceOTU2455 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| 278303 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| NewCleanUpReferenceOTU155365 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |

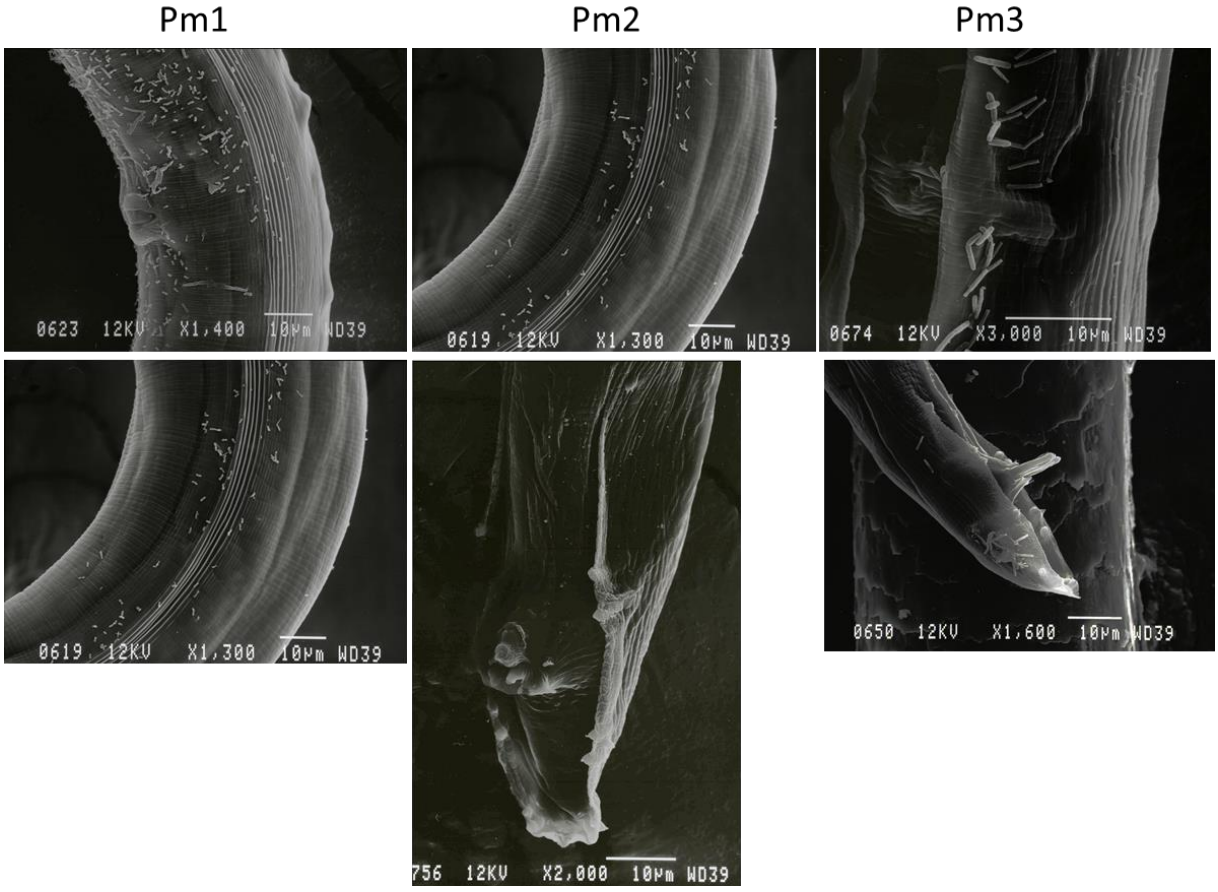
| | |
|---|---|
| NewReferenceOTU2438 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga |
| NewReferenceOTU2438 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| NewCleanUpReferenceOTU29602 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| NewReferenceOTU4131 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| NewReferenceOTU2305 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| 147086 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| NewCleanUpReferenceOTU155365 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| NewReferenceOTU2455 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| 278303 | Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriaceae.gCellulophaga.slytica |
| 4313779 | Bacteroidetes.Saprospirae |
| NewReferenceOTU631 | Bacteroidetes.Saprospirae |
| NewReferenceOTU2357 | Bacteroidetes.Saprospirae |
| NewReferenceOTU631 | Bacteroidetes.Saprospirae.Saprospirales |
| NewReferenceOTU2357 | Bacteroidetes.Saprospirae.Saprospirales |
| 4313779 | Bacteroidetes.Saprospirae.Saprospirales |
| NewReferenceOTU2357 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae |
| NewReferenceOTU631 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae |
| 4313779 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae |
| NewReferenceOTU631 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g |
| 4313779 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g |
| NewReferenceOTU2357 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g |
| NewReferenceOTU2357 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g.s |
| NewReferenceOTU631 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g.s |
| 4313779 | Bacteroidetes.Saprospirae.Saprospirales.Saprospiraceae.g.s |
| NewReferenceOTU2110 | Proteobacteria |
| 160928 | Proteobacteria |
| 530962 | Proteobacteria |
| NewReferenceOTU2428 | Proteobacteria |
| NewCleanUpReferenceOTU73063 | Proteobacteria |
| NewReferenceOTU2190 | Proteobacteria |
| NewReferenceOTU1394 | Proteobacteria |
| NewCleanUpReferenceOTU249675 | Proteobacteria |
| 820978 | Proteobacteria |
| 198609 | Proteobacteria |
| 346035 | Proteobacteria |
| NewReferenceOTU2279 | Proteobacteria |
| 4304357 | Proteobacteria |
| 785565 | Proteobacteria |
| NewReferenceOTU315 | Proteobacteria |
| NewCleanUpReferenceOTU249675 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU315 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU73063 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU1394 | Proteobacteria.Alphaproteobacteria |
| NewReferenceOTU2110 | Proteobacteria.Alphaproteobacteria |

| | |
|---|---|
| 4304357 | Proteobacteria.Alphaproteobacteria |
| NewCleanUpReferenceOTU249675 | Proteobacteria.Alphaproteobacteria.Rhizobiales |
| NewCleanUpReferenceOTU249675 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae |
| NewCleanUpReferenceOTU249675 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae.gAgrobacterium |
| NewCleanUpReferenceOTU249675 | Proteobacteria.Alphaproteobacteria.Rhizobiales.Rhizobiaceae.gAgrobacterium.s |
| NewReferenceOTU315 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewCleanUpReferenceOTU73063 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU2110 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU1394 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| 4304357 | Proteobacteria.Alphaproteobacteria.Rhodobacterales |
| NewReferenceOTU315 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU2110 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 4304357 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewReferenceOTU1394 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| NewCleanUpReferenceOTU73063 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae |
| 4304357 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU315 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU2110 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewCleanUpReferenceOTU73063 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g |
| NewReferenceOTU2110 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| 4304357 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewCleanUpReferenceOTU73063 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU315 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.g.s |
| NewReferenceOTU1394 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gPhaeobacter |
| NewReferenceOTU1394 | Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae.gPhaeobacter.s |
| NewReferenceOTU2428 | Proteobacteria.Gammaproteobacteria |
| 346035 | Proteobacteria.Gammaproteobacteria |
| NewReferenceOTU2279 | Proteobacteria.Gammaproteobacteria |
| 820978 | Proteobacteria.Gammaproteobacteria |
| 160928 | Proteobacteria.Gammaproteobacteria |
| 198609 | Proteobacteria.Gammaproteobacteria |
| NewReferenceOTU2190 | Proteobacteria.Gammaproteobacteria |
| 530962 | Proteobacteria.Gammaproteobacteria |
| 785565 | Proteobacteria.Gammaproteobacteria |
| 160928 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 198609 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 530962 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU2279 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU2190 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 785565 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 820978 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| NewReferenceOTU2428 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 346035 | Proteobacteria.Gammaproteobacteria.Vibrionales |
| 820978 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |

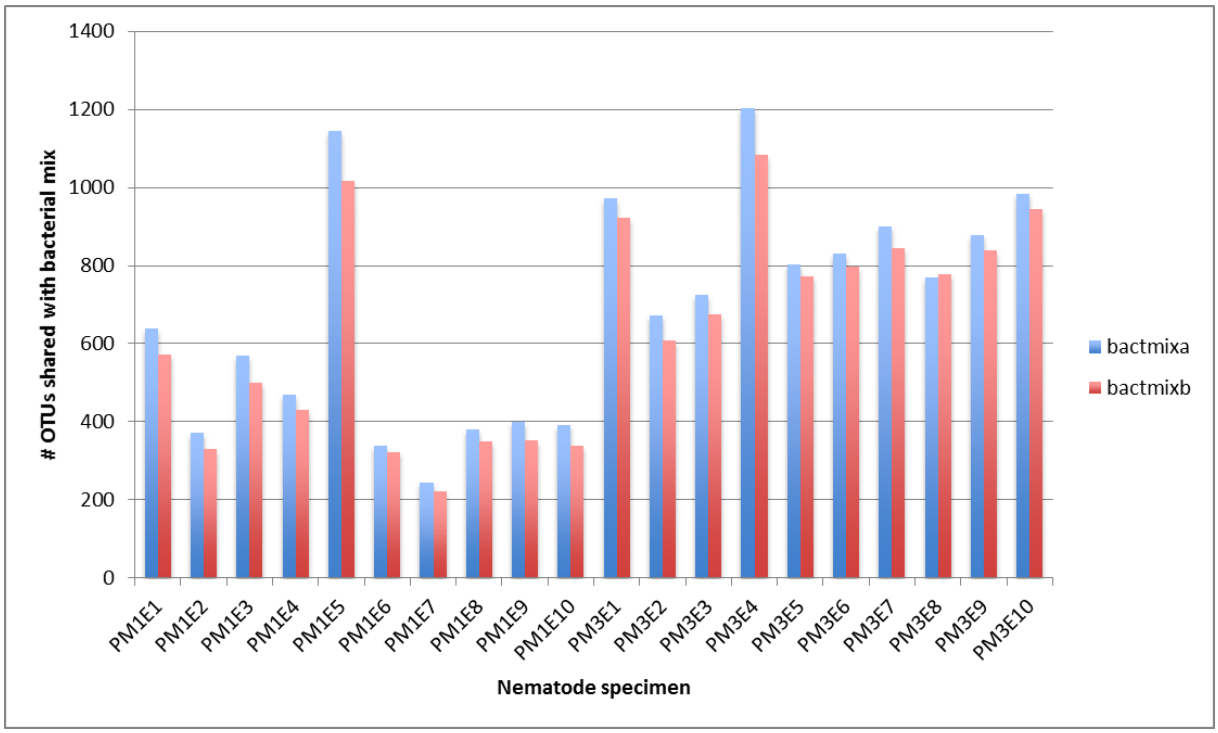
| | |
|---|---|
| NewReferenceOTU2428 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 530962 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 785565 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 198609 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae |
| 198609 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g |
| 785565 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g |
| 820978 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g |
| NewReferenceOTU2428 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g |
| 530962 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g |
| 198609 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g.s |
| 530962 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g.s |
| 785565 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g.s |
| 820978 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g.s |
| NewReferenceOTU2428 | Proteobacteria.Gammaproteobacteria.Vibrionales.Pseudoalteromonadaceae.g.s |
| 346035 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| 160928 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewReferenceOTU2279 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewReferenceOTU2190 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae |
| NewReferenceOTU2279 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio |
| 346035 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio |
| 160928 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio |
| NewReferenceOTU2190 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio |
| 346035 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio.s |
| 160928 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio.s |
| NewReferenceOTU2279 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio.s |
| NewReferenceOTU2190 | Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.gVibrio.s |
| NewReferenceOTU1759 | Unassigned |
| NewReferenceOTU1875 | Unassigned |
| NewReferenceOTU1504 | Unassigned |
| NewReferenceOTU1550 | Unassigned |
| NewReferenceOTU1045 | Unassigned |
| NewReferenceOTU2401 | Unassigned |
| NewReferenceOTU2750 | Unassigned |
| NewReferenceOTU54 | Unassigned |
| NewReferenceOTU3705 | Unassigned |
| NewReferenceOTU3574 | Unassigned |
| NewReferenceOTU2014 | Unassigned |
| NewCleanUpReferenceOTU101342 | Unassigned |
| NewCleanUpReferenceOTU216626 | Unassigned |
| NewCleanUpReferenceOTU83057 | Unassigned |
| NewReferenceOTU1877 | Unassigned |
| NewReferenceOTU1507 | Unassigned |
| NewReferenceOTU1172 | Unassigned |
| NewReferenceOTU2313 | Unassigned |

| | |
|---|---|
| NewReferenceOTU2420 | Unassigned |
| NewReferenceOTU1043 | Unassigned |
| NewReferenceOTU1848 | Unassigned |
| NewReferenceOTU3347 | Unassigned |
| NewReferenceOTU949 | Unassigned |
| NewCleanUpReferenceOTU236908 | Unassigned |
| NewReferenceOTU2197 | Unassigned |
| NewCleanUpReferenceOTU201181 | Unassigned |
| NewReferenceOTU2024 | Unassigned |
| NewReferenceOTU2369 | Unassigned |
| NewReferenceOTU3153 | Unassigned |
| NewReferenceOTU2168 | Unassigned |
| NewCleanUpReferenceOTU177381 | Unassigned |
| NewReferenceOTU1376 | Unassigned |
| NewReferenceOTU3457 | Unassigned |
| NewReferenceOTU1442 | Unassigned |
| NewReferenceOTU3095 | Unassigned |
| NewReferenceOTU1675 | Unassigned |
| NewReferenceOTU2684 | Unassigned |
| NewReferenceOTU1502 | Unassigned |
| NewReferenceOTU2448 | Unassigned |
| NewReferenceOTU1057 | Unassigned |
| NewReferenceOTU3078 | Unassigned |
| NewReferenceOTU3922 | Unassigned |
| NewCleanUpReferenceOTU147402 | Unassigned |
| NewReferenceOTU1787 | Unassigned |
| NewReferenceOTU1236 | Unassigned |
| NewReferenceOTU1493 | Unassigned |
| NewReferenceOTU2537 | Unassigned |
| NewReferenceOTU1422 | Unassigned |
| NewReferenceOTU1042 | Unassigned |
| NewReferenceOTU1160 | Unassigned |
| NewReferenceOTU1756 | Unassigned |
| NewReferenceOTU1872 | Unassigned |

**Appendix S12:** SEM pictures of nematode specimens from Pm1, Pm2 and Pm3 with bacteria attached to the cuticula. Top row: vulva region of the females (laterofrontal view for Pm1, lateral view for Pm2 and ventral view for Pm3); bottom row: males. For Pm1, this is a lateral view of the mid body region, for Pm2 a ventral view of the tail posterior region with bursa and spicula and for Pm3 a lateral view of the posterior body region with ejected spicula.

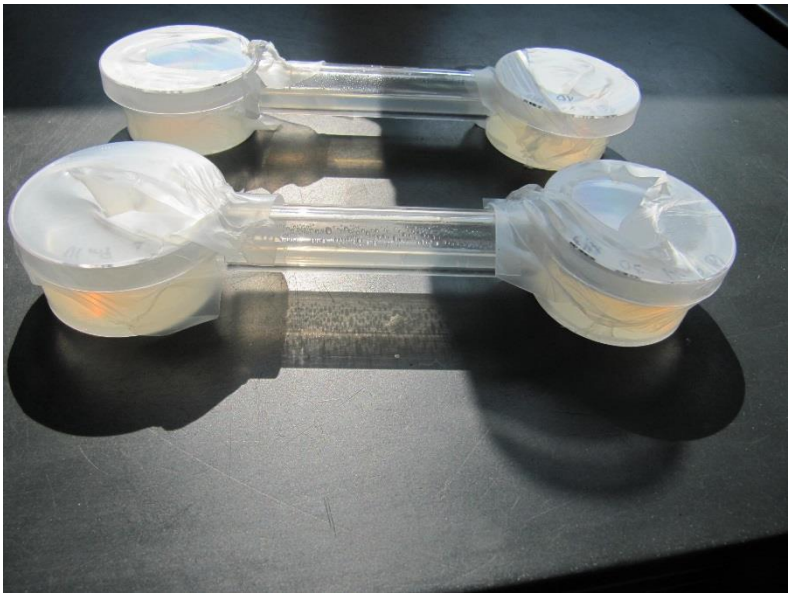


**Appendix S13:** Number of shared OTUs between each nematode specimen and each replica of the bacterial mix.



# 6

# CHAPTER VI



# DISPERSAL DIFFERENCES AMONG CRYPTIC SPECIES

---

Slightly modified from:

De Meester, N., Derycke, S. & Moens, T. (2012) Differences in time until dispersal between cryptic species of a marine nematode species complex. *PloS one*, 7, e42674.

## Abstract

Co-occurrence of closely related species may be achieved in environments with fluctuating dynamics, where competitively inferior species can avoid competition through dispersal. Here we present an experiment in which we compared active dispersal abilities (time until first dispersal, number and gender of dispersive adults, and nematode densities at time of dispersal) in *Litoditis "marina"*, a common bacterivorous nematode species complex comprising four often co-occurring cryptic species, Pm I, II, III and IV, as a function of salinity and food distribution. The experiment was conducted in microcosms consisting of an inoculation plate, connection tube and dispersal plate. Results show species-specific dispersal abilities with Pm I dispersing almost one week later than Pm III. The number of dispersive adults at time of first dispersal was species-specific, with one dispersive female in Pm I and Pm III and a higher, gender-balanced, number in Pm II and Pm IV. Food distribution affected dispersal in a species-specific way: in absence of food in the inoculation plate, Pm I start dispersing as fast as the other species, so all species dispersed after ca. four days. When food was present in the inoculation plate Pm I dispersed later (compared with the treatments where no food was present in the inoculation plate), but at the same time and densities irrespective of food conditions in the dispersal plate, suggesting density-dependent dispersal. Pm III dispersed not only faster than Pm I, but also at a lower population density. Salinity affected dispersal, with slower dispersal at higher salinity. These results suggest that active dispersal in *Litoditis "marina"* is common, density-dependent and with species, gender- and environment-specific dispersal abilities. These differences can lead to differential responses under suboptimal conditions and may help to explain temporary coexistence at local scales.

## Introduction

Biodiversity in many ecosystems appears significantly higher than previously thought due to cryptic genetic diversity which underlies a broad range of morphospecies (Bickford, et al., 2007; Pfenninger & Schwenk, 2007). Despite increasing documentation of cryptic diversity, knowledge about the ecology of cryptic species remains very scant (Ralin, 1968; Gerhardt, 2005; Rissler & Apodaca, 2007; Alizon, et al., 2008; De Meester, et al., 2011 (chapter II)). Morphologically highly similar species may show high functional similarity and niche overlap (Winston, 1995; Ortells, et al., 2003; Zhang, et al., 2004) which seems at odds with traditional competition theory (Darwin, 1859; Webb, et al., 2002; Violle, et al., 2011).

Coexistence of closely related species can be achieved in environments with fluctuating dynamics in time or space (e.g. presence of other species, food distribution). Here, competitively inferior species may persist because they are temporarily favoured by specific conditions (Begon, et al., 1996). Alternatively, species that are sufficiently motile can move to suitable patches and thus avoid competition (Snyder & Chesson, 2003). In this way, they can at least temporarily achieve some form of coexistence but escape from it through small-scale dispersal when competitive pressure becomes too strong. This movement of individuals away from their natal environment is dispersal and can lead to gene flow over different spatial scales (Ronce, 2007; Gienapp, et al., 2008). Dispersal is a process, triggered partly by the intrinsic condition of organisms, such as gender, competitive ability, genetic variability and species identity (Ólafsson & Moore, 1990; Ims & Hjermann, 2001; Schratzberger, et al., 2004; Ullberg, 2004; Guilini, et al., 2011), and partly by environmental conditions, such as habitat and food quality, population density and intraspecific interactions (Lee, et al., 1977; Neira, et al., 2001; Bowler & Benton, 2005; Harvey, 2009).

In contrast with most larger marine benthic vertebrates having at least one life stage in which dispersal occurs on a specific spatial scale (Ronce, 2007), most meiobenthic species (nematodes and other small metazoans in the size range of 0.04 to 2 mm (Mare, 1942)) lack a pelagic stage and have long been considered poor dispersers due to their small size and poor swimming ability (Palmer, 1988). Nematodes are the most abundant meiofauna in marine sediments (Heip, et al., 1985; Coomans, 2000) and have a high species diversity at both global and local scales (Lambshead & Boucher, 2003). They can passively disperse following erosion from sediments or through rafting on algae ((Thiel & Gutow, 2005), but they can also actively enter the water column (Ullberg, 2004), which may facilitate both

small-scale active dispersal as well as larger-scale passive dispersal (Chandler & Fleeger, 1983; Armonies, 1988). They can at least partly control their settlement back to the sediment (Ullberg & Olafsson, 2003). In addition, they migrate laterally through sediments (Schratzberger, et al., 2004; Ullberg, 2004), but the rates and distances over which nematodes actively disperse and the extrinsic and intrinsic drivers of dispersal remain poorly known. Salinity effects, for instance, have not been tested before, probably because the effect of salinity variation has mostly been considered on a broader geographical scale. However, salinity can even vary between two nearby algae patches because of variation in shallow gullies and puddles. Moreover, diurnal (Kaiser, et al., 2005) and seasonal variations (Tietjen & Lee, 1972) in salinity also occur. These salinity differences may affect small-scale dispersal of meiofauna in a direct or indirect way (Ims & Hjermann, 2001).

*Litoditis "marina"* (Sudhaus, 2011) is a common bacterivorous nematode associated with decomposing macroalgae in the littoral zone of coastal and estuarine environments (Inglis & Coles, 1961; Sudhaus, 1974). Several cryptic species have been found within this morphospecies, formerly known as *Rhabditis marina* or *Pellioditis marina* (Derycke, et al., 2008a), four (Pm I, Pm II, Pm III and Pm IV) of which frequently occur along the south-western coast and estuaries of The Netherlands (Derycke, et al., 2008b). Moreover, it is common to find two or three of these cryptic species co-occurring (Derycke, et al., 2006). These species show concordant molecular divergences at nuclear and mitochondrial loci but lack single distinctive morphological differences (Derycke, et al., 2008a; Fonseca, et al., 2008), and crossbreeding between them does not occur (Fonseca, et al., 2008). All species are gonochoristic; parthenogenesis has hitherto not been observed (Tietjen & Lee, 1972). Females of Pm I and IV largely reproduce through vivipary, whereas Pm II and Pm III use ovipary. They produce several tens up to 600 progeny per female (Vranken & Heip, 1983; Moens & Vincx, 2000b; dos Santos, et al., 2008), the vast majority of which are released during the first few days following maturation to adults. All four species have minimum juvenile development times of ca. 4 days at temperatures around 20°C, salinities between 15 and 30, and sufficient food availability (De Meester, et al., 2015a; chapter III). Both geographical and seasonal variation in abundance and dominance of these cryptic species occurs (Derycke, et al., 2006) and may be linked to environmental variation (e.g. salinity). Recent laboratory experiments have also demonstrated that salinity affects the outcome of competitive and facilitative interactions between these cryptic species, with competition

being more pronounced at lower salinity and Pm IV and Pm II being competitively inferior to Pm I and Pm III (De Meester, et al., 2011; chapter II).

In the present study, we tested if differences in dispersal abilities between the four different cryptic species of *L. "marina"* exist and if these differences are gender- and environment-specific. In a first experiment the effects of food distribution on the dispersal ability of cryptic species are tested. If active dispersal exists in the cryptic species, we expect that it will occur more when food is limited in the source patch but still available in nearby patches. In a second experiment dispersal abilities of the four cryptic species were tested at different salinities. A previous experiment already showed that population growth and competitive ability of the cryptic species differed between two salinities (De Meester, et al., 2011; chapter II). At the lower salinity, population growth rate of Pm III and Pm IV was higher, suggesting that this salinity was more favourable. We thus expected that this faster population growth would result in more pronounced intraspecific competition, leading to faster dispersal at the lower salinity (Thomas & Jepson, 1999; Saastamoinen & Hanski, 2008). These experiments will yield insight in extrinsic (salinity and food distribution) and intrinsic (species identity and gender) factor-dependent dispersal. Investigating the dispersal abilities of species is crucial to understand the highly dynamic patterns and the ecology of meiobenthic communities (Commito & Tita, 2002) and the resilience of populations under fluctuating environmental conditions (Harrison, 1979).

## Materials & Methods

### Nematode cultures

Monospecific cultures of the four different cryptic species were raised from one single gravid female per species and maintained on sloppy (1%) nutrient:bacto agar media (Moens & Vincx, 1998) (temperature of 20°C; salinity of 25) with unidentified bacteria from their habitat as food. Species identity and monospecificity of stock cultures were tested shortly after their initiation and on regular moments thereafter on several individuals with a species-specific qPCR assay using ITS sequences (Derycke, et al., 2012). Nematodes for the experiments were harvested from these stock cultures in exponential growth phase.

### Dispersal experiments

Dispersal abilities of the cryptic species were measured as time until the first effective dispersal event. Dispersal was considered effective if it was followed by reproduction in the

dispersal plate, regardless whether the individual was already gravid before the dispersal event. To study the differences in time until dispersal between the four cryptic species, specially designed dispersal plates were used (Fig. 1). These plates consist of two Petri dishes (each 5 cm i.d.; an 'inoculation' plate and a 'dispersal' plate, respectively) connected by a tube (1 cm i.d. and 10 cm length). The length of this test tube was based on results of a preliminary test with tubes of various lengths. Considerably shorter tube lengths resulted in almost instantaneous migration to the dispersal plate, through random movement and/or through direct chemotaxis to food on the dispersal plate. Longer tube lengths (≥15 cm) resulted in very slow dispersal irrespective of presence of food in the dispersal plate. The substratum in the plates was provided as 60 mL of a 1.5% bacto agar medium prepared with artificial seawater (Dietrich & Kalle, 1957). The agar was spread equally over the two different plates and the connection tube taking care that the surface was at the same level and continuous in both plates and connection tube. The relatively high concentration of the agar (1.5%) hampers burrowing of nematodes into the agar and thus restricts their movement to the agar surface, which greatly facilitates observations. The pH of the agar medium was buffered at 7.5 – 8 with TRIS-HCl in a final concentration of 5mM. The addition of the buffer increases the initial salinity by ca 1.2 units. Two sets of dispersal experiments were performed, the first focusing on the role of food availability in the dispersal and inoculation plates, the second focusing on the effect of salinity on dispersal.

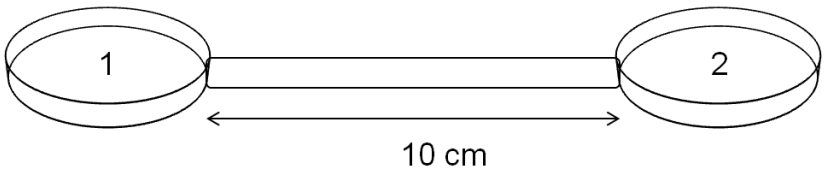



Figure 1: Design of the dispersal microcosms with plate 1 being the inoculation plate and plate 2 the dispersal plate. Dispersal ability was scored when nematodes first arrived at plate 2.

*A. Food distribution experiment*

The experiment was started by manually picking up five adult males and five adult females from the stock cultures of a single cryptic species. Before placing the organisms randomly in the inoculation plate, they were bathed in clean artificial seawater (salinity of 25) for 2 h to remove most adhering bacteria. For every cryptic species, four different treatments were used. In the 'B' treatment food was added to both plates (inoculation and dispersal plate); in the 'I' treatment food was only added to the inoculation plate and not to the dispersal plate; in the 'D' treatment food was only added to the dispersal plate, and in the 'N' treatment food was absent from both plates. Food consisted of frozen-and-thawed *Escherichia coli* (strain K12) and was added every eighth day, well before food depletion occurred (50 µL of a suspension with a density of $3x10^9$ cells $mL^{-1}$) (dos Santos, et al., 2008). This bacterial strain has been shown to be a suitable food source for cultures of these four cryptic species of *L. "marina"*. No food was ever added to the connection tube, but dispersing nematodes do carry bacteria on their cuticles and thus spread some food into the connection tube and dispersal plate. All plates contained agar with a salinity of 25 and were incubated in the dark at a constant temperature of 20°C. There were four independent replicates per treatment.

Population and dispersal dynamics were studied by counting adults and juveniles every day in both plates. The timing of the arrival of the first organism at the dispersal plate was recorded, as well as the life stage (adult or juvenile) and the effectiveness of the dispersal event. Observations on the organisms in the connection tube and on nematode tracks were made, to verify if organisms were moving from the inoculation to the dispersal plate and not in a random way. Moreover, the gender of the first dispersers was recorded for the B treatment. After 20 days, the experiment was stopped because the agar medium started to become liquid. By that time, dispersal had occurred in every replicate.

*B. Salinity experiment*

Additional dispersal plates were started with food in both plates (treatment B, see above), but with agar medium with a salinity of 15 instead of 25. Methods and incubation conditions were the same as described for the food distribution experiment.

**Data analyses**

Differences in the time until first effective dispersal between the cryptic species and between food treatments were tested in R (R Development Core Team, 2008) with a two-way ANOVA (species and food distribution as independent variables), as the assumptions for parametric tests were met. Abundances of adults, juveniles and total nematodes in the inoculation plate at the moment of first effective dispersal were also compared between the different species and food distributions by using a two-way ANOVA. A Tukey HSD test was used for posterior pair wise comparisons. A log transformation on the adult abundances in the dispersal plate at first dispersal event was used, as the data were not normally distributed. The analyses for the dispersive organisms were conducted with the data of the adults only. Juveniles were omitted from the analyses, as Pm I and Pm IV are viviparous species and in this way it was not possible to determine whether the juveniles present in the dispersal plate were real dispersers or offspring of dispersed adults.

Differences in the time until first effective dispersal between the cryptic species and between salinities were also tested with ANOVA, and so were the abundances of adults, juveniles and total nematodes in the inoculation plate at the moment of dispersal, and the number of dispersive adults. When no significant interaction effects were found, one-way ANOVA or Kruskal-Wallis tests within one species were conducted to look for the effect of salinity within each species separately.

Differences in gender-specificity of dispersal between the cryptic species was tested by calculating the proportion of females at the first dispersal event and conducting a Kruskal-Wallis test with proportion of females as dependent variable and species as independent variable, as the assumptions for parametric tests were not met.

# Results

### Food effects on time until dispersal between the different cryptic species

Time until first effective dispersal between the four cryptic species of *L. "marina"* was influenced by the interaction effect between species and food distribution (Table 1a). Food distribution had a pronounced effect on the time until dispersal of Pm I, with a significantly longer time until dispersal for the B and the I treatment (dispersal occurred respectively after 14.5 ± 1.6 days and 14.8 ± 1.9 days) compared with the D and N treatment (resp. average of 5 ± 0.9 and 6.5 ± 0.3 days until dispersal, fig 2a). In the B and I treatment, Pm I also dispersed more slowly than the other species, except for Pm I in the B treatment compared with Pm IV in the B treatment and with Pm III in the N treatment (for B treatment: 6.3 ± 1.0 days (Pm II), 4.3 ± 2.0 days (Pm III); for I treatment: 5.8 ± 1.0 days (Pm II), 5.5 ± 0.9 days (Pm III) and 7.0 ± 0.4 days (Pm IV); for N treatment: 6.5 ± 0.5 days (Pm II) and 6.5 ± 0.5 days (Pm IV)). Time until dispersal for the D treatment did not differ between the species and no differences between the different food treatments were found for the other species (Fig. 2a).

Table 1: Results of the two-way ANOVA (independent factors: species (Pm I, Pm II, Pm III and Pm IV) and food treatment (B,D,N,I)) on a) time until dispersal and number of dispersive adults and b) total, adult and juvenile density in the inoculation plate at the first dispersal event.

| **A)** | | **Time until dispersal** | | **Dispersive adults** | |
|---|---|---|---|---|---|
| | df | F | p | F | p |
| Species | 3 | 13.56 | **< 0.0001** | 4.70 | **0.0059** |
| Food | 3 | 10.47 | **< 0.0001** | 1.18 | 0.33 |
| Species:Food | 9 | 4.89 | **0.00012** | 3.11 | **0.0050** |

| **B) Density in inoculation plate** | | **Total** | | **Adult** | | **Juvenile** | |
|---|---|---|---|---|---|---|---|
| | df | F | p | F | p | F | p |
| Species | 3 | 0.49 | 0.69 | 3.06 | **0.037** | 0.80 | 0.50 |
| Food | 3 | 11.40 | **< 0.0001** | 10.36 | **< 0.0001** | 9.63 | **< 0.0001** |
| Species:Food | 9 | 1.63 | 0.13 | 2.16 | **0.042** | 1.42 | 0.21 |

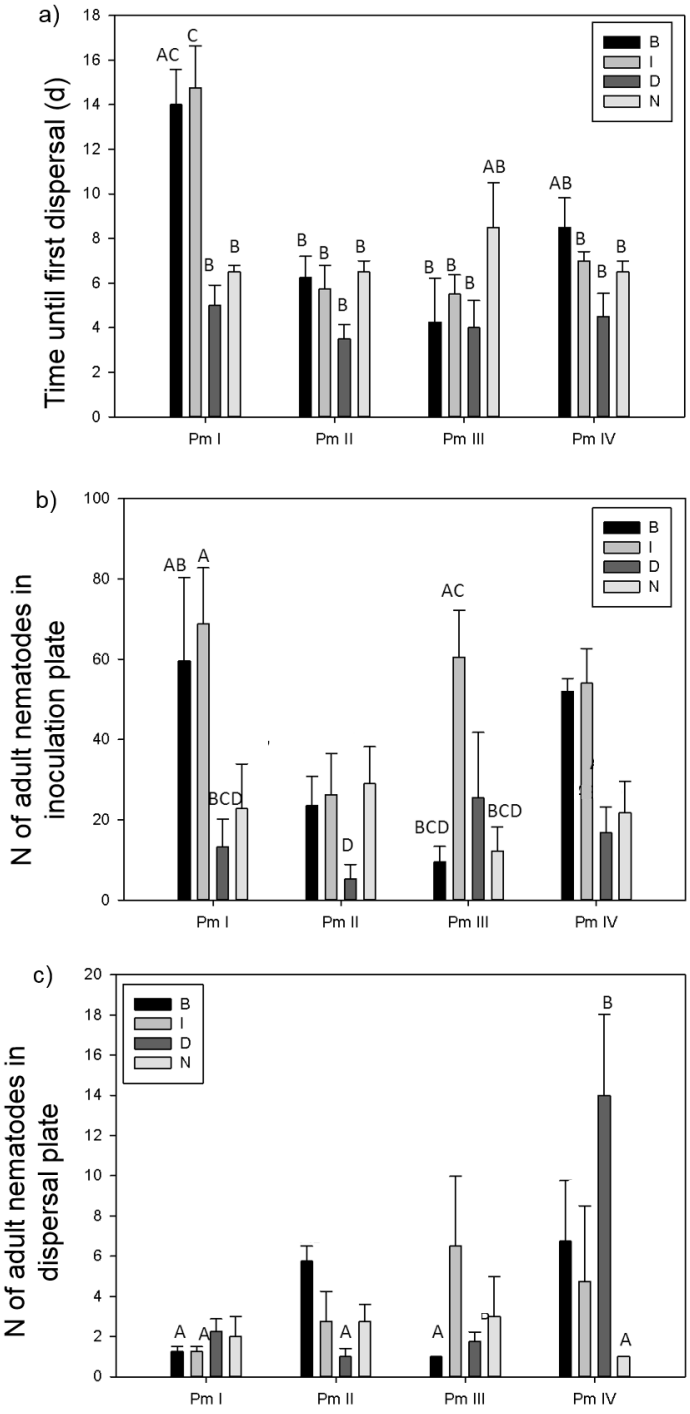



Figure 2. Effect of food distribution on dispersal abilities: (a) average time until first dispersal event (mean ± SE), (b) average number of adults in the inoculation plate (mean ± SE) at time of first dispersal event and (c) average number of adults in the dispersal plate (mean ± SE) at time of first dispersal for the four cryptic species of *L. "marina"* at four different food treatments. (B: food at inoculation and dispersal plate; I: only food in inoculation plate; D: only food in dispersal plate; N:no food in both plates) (letters above bars indicate pairwise significant differences; $p<0.05$; $n = 64$; the absence of letters means that there were no significant differences with any of the other treatments).

Total nematode density in the inoculation plate at the moment of first effective dispersal only differed between the different food distribution treatments (Table 1b), with significant differences between the I treatment and the three other treatments. Dispersal occurred at the lowest nematode density for the D treatment (47.6 ± 15.2 organisms over the four species), followed by the N treatment (114.7 ± 26.5 organisms), the B treatment (119.1 ± 29.8 organisms) and the I treatment (215.8 ± 23.7 organisms). Food distribution had the same effect on juvenile and adult densities in the inoculation plate at first dispersal (Table 1b). In addition, adult numbers in the inoculation plate differed between species , and a significant interaction effect between food distribution and species was observed (Table 1b, Fig. 2b), with lower adult numbers at time of first dispersal for Pm II in the D treatment (5.3 ± 3.6 adults) compared with Pm I in the B and I treatment (resp. 59.5 ± 20.9 adults and 68.8 ± 10.2 adults) and Pm III in the I treatment (60.5 ± 11.7 adults).

For number of dispersive adults at time of first effective dispersal the interaction between food distribution and species was significant (Table 2a). Significant differences between species were also found mostly the result of a higher number of dispersive adults for Pm IV in the D treatment (14 ± 4.02 adults) compared with Pm II in this treatment (1.0 ± 0.5), Pm I in the B and I treatment (resp. 1.3 ± 0.3 and 1.3 ± 0.4 adults), Pm III in the B treatment (1.0 ± 0.0 adults) and Pm IV in the N treatment (1.0 ± 0.0 adults) (Fig. 2c).

**Salinity effects on time until dispersal between the different cryptic species**

Time until first effective dispersal was shorter at a lower salinity for all species (Table 2), with an average of 5.8 ± 1.1 days at a salinity of 15 compared to 8.4 ± 2.1 days at a salinity of 25. Time until dispersal also differed between the four cryptic species over the two salinities (Table 2) with Pm I again being the slowest disperser. Dispersal in Pm I occurred only after 11.4 ± 1.5 days (average over the two salinities) compared to 5.5 ± 0.5 days in Pm II, 3.9 ± 0.97 days in Pm III and 7.4 ± 0.8 days in Pm IV (Fig. 3a). No interaction effect between species and salinities was found (Table 2), indicating that the salinity effect was similar for all four cryptic species. However, no significant differences in time until first dispersal within species could be found ($F_{1,6}$ , all $P > 0.05$).

Total numbers of organisms in the inoculation plate at time of first effective dispersal were lower at a salinity of 15 than at a salinity of 25 (77.3 ± 15.3 vs. 126.7 ± 16.1 organisms; table 2) over the four cryptic species. Total abundances at time of dispersal were also species-

specific (Table 2), with Pm III dispersing at much lower total densities (28.1 ± 12.0 organisms) compared with the three other species (resp. 139.4 ± 13.3 organisms (Pm I), 92.6 ± 30.1 organisms (Pm II) and 117.9 ± 21.3 organisms (Pm IV)) over the two salinities. The same trend was found when focusing on abundances of juveniles (Table 2). Adult abundances at first dispersal were also species-specific, with significant differences between Pm III (6.1 ± 2.3 adults) compared with Pm I and Pm IV (resp. 57.6 ± 11.4 and 43.5 ± 7.5 adults), but no effect of salinity could be found (Table 2, Fig. 3b). An interaction effect between salinity and species was absent in all three cases ($F_{3,24}$, all P> 0.32). For total nematode density, significant differences within one species were found for Pm III ($F_{1,6}$= 7.74 , P= 0.032) and Pm IV ($F_{1,6}$= 14.97 , P= 0.0083), with higher total abundances at a salinity of 25 (52.0 ± 17.0 (Pm III) and 165.5 ± 24.0 (Pm IV)) than at a salinity of 15 (4.25 ± 2.6 (Pm III) and 70.3 ± 5.6 (Pm IV)). No significant differences were found within one species for the adult nematode density ($F_{1,6}$ , all P > 0.05).

Table 2: Results of the two-way ANOVA (independent factors: species (Pm I, Pm II, Pm III and Pm IV) and salinity treatment (15 and 25) on time until dispersal and total, adult and juvenile densities in the inoculation plate at first time of dispersal.

| | time until dispersal | | total density | | adult density | | juvenile density | |
|---|---|---|---|---|---|---|---|---|
| **ANOVA** | df | F | p | F | p | F | p | F | p |
| Species | 3 | 12.86 | **< 0.0001** | 6.91 | **0.0021** | 5.7 | **0.0041** | 4.30 | **0.014** |
| Salinity | 1 | 7.32 | **0.012** | 5.49 | **0.028** | 0.41 | 0.52 | 6.80 | **0.016** |
| Species:Food | 3 | 1.20 | 0.33 | 0.32 | 0.81 | 1.22 | 0.32 | 1.42 | 0.94 |

Numbers of adults in the dispersal plate at time of first dispersal did not differ between the different salinity treatments, but did differ between the different species (ANOVA, $F_{3,24}$=4.81, P= 0.0091), with higher numbers of dispersive organisms for Pm II (5.4 ± 0.8 adults) than for Pm III (1.3 ± 0.2 adults; P= 0.03) (Fig. 3c). One-way ANOVA's between salinities within one species did not reveal any differences ($F_{1,6}$ , all P > 0.05).

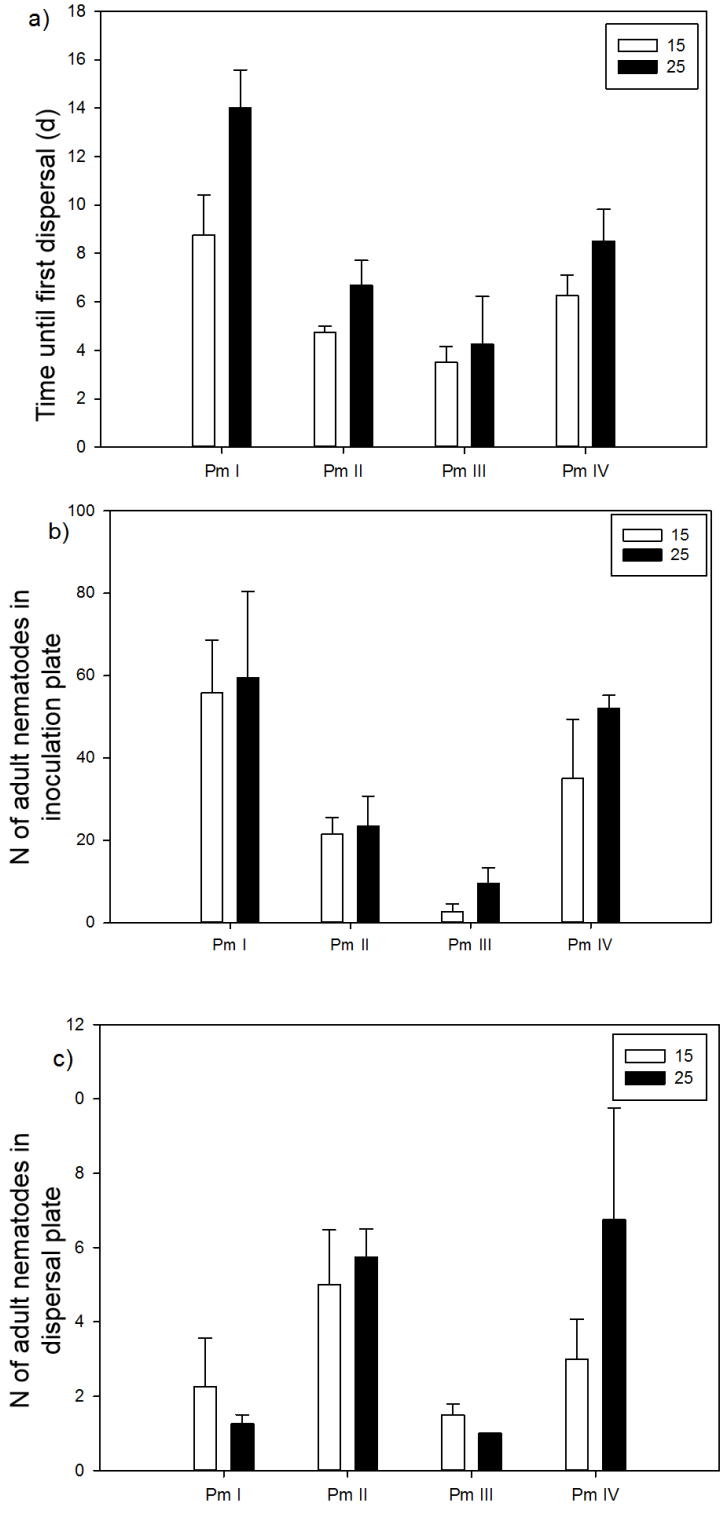



Figure 3: Effect of salinity on dispersal abilities: (a) average time until first dispersal event (mean ± SE), (b) average number of adults in the inoculation plate (mean ± SE) at time of first effective dispersal and (c) average number of adults in the dispersal plate at the first dispersal event (mean ± SE) for the four cryptic species of *L. "marina"* at two different salinities (no pairwise significant differences were found; $p>0.05$; $n = 32$).

**Gender effect on dispersal**

Proportion of females among the first dispersive nematodes differed between the different cryptic species (Kruskal-Wallis, H= 11.27, P= 0.01), with consistently only females being the first dispersers in Pm I and Pm III. A more balanced ratio of dispersive females and males was found for Pm II and Pm IV (Fig. 4).

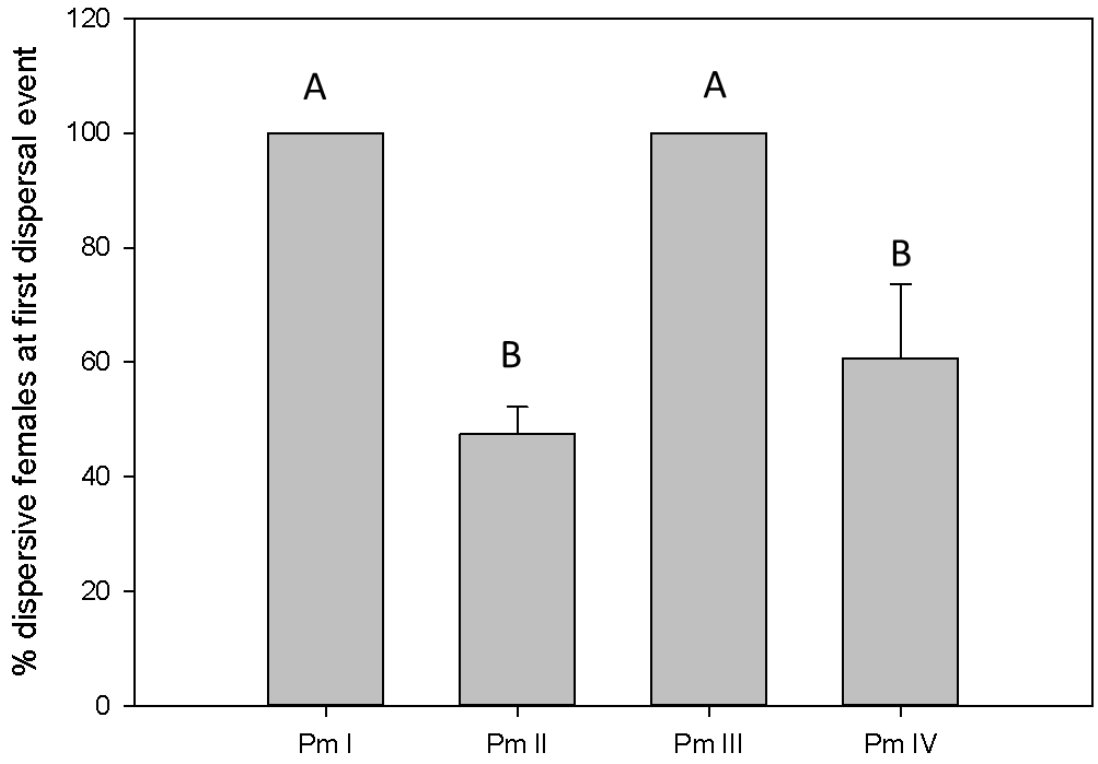



Figure 4: Proportion of females among dispersive adult nematodes at first dispersal (mean ± SE) for the four cryptic species of *L. "marina"* in the B treatment (letters indicate significant differences; $p < 0.05$ ; $n = 16$). Note that an error bar is missing for Pm I and Pm III because in all replicates one female was the first disperser.

## Discussion

To date, there is only limited evidence for differential dispersal in meiofauna at the level of species (Wetzel, et al., 2002; Ullberg & Olafsson, 2003; Boeckner, et al., 2009; Thomas & Lana, 2011), particularly with respect to active dispersal. Three important characteristics explaining differential dispersal rates in benthic nematode assemblages are size (Gallucci, et al., 2008), life history (Zhou, 2001; Gallucci, et al., 2008) and vertical position inside the sediment (Schratzberger, et al., 2004). In addition, even closely related nematode species may differentially disperse towards different food patches (Moens, et al., 1999; Höckelmann, et al., 2004).

**Species-specific dispersal abilities exist within the cryptic species complex**

In our experiment, active dispersal occurred in all four cryptic species of *L. "marina"* in less than two weeks, and significant differences between the species were observed. Pm I was the slowest disperser, taking almost one week longer to disperse than Pm III, the fastest disperser. It is unlikely that any of the above mentioned factors can explain the observed differences in time until dispersal in our study. Size differences between the cryptic species are limited (Fonseca, et al., 2008). The little information available on life history differences between these cryptic species (increased population growth for Pm III and Pm IV at a salinity of 15 (De Meester, et al., 2011; chapter II)), suggests that such differences are rather subtle, and do not clearly correlate with the dispersal differences observed here. Moreover, *L. "marina"* is not a true infaunal species but rather frequents patches of decomposing algae or biofilms on living algae, rendering a direct link between position in the substratum and dispersal unlikely. Finally, the food conditions were the same for all four species in the present experiments. The different dispersal ability of Pm I may, however, be related to species-specific attraction to food sources. In a preliminary experiment on the migration of these four cryptic species towards different bacterial strains, Pm I was the only species which readily moved towards *E. coli* (Derycke, unpublished data). This is surprising given the fact that lab cultures of all four species are easily maintained on *E. coli*, but these results could explain why Pm I dispersed sooner in the D treatment compared with the B and I treatments (Fig. 2a). At the same time, if Pm I has a stronger preference for *E. coli* as food than the other cryptic species, then this might also explain why Pm I generally dispersed later than the other species in the B and I treatments, but it does not explain why dispersal was equally fast when no food was available in both plates and thus no food trigger was present (N treatment) as in the D treatment. In the N and D treatment, time of first dispersal was no

longer species-specific and occurred around the fourth day in all species, probably to avoid the suboptimal conditions of the inoculation plate (no food). This shows that Pm I is able to disperse faster under certain food conditions and time until dispersal is not merely the result of behavioural differences in activity or motility between different cryptic species. Nematodes were also able to survive and even reproduce in plates without food, probably because they survive temporarily on energy reserves and nematodes, even after washing, still carry some bacteria from the stock cultures on their cuticles and thus spread some food even in treatments where none had been inoculated (N treatment).

**Importance of density-dependent dispersal**

For Pm I and Pm IV organisms disperse when densities become too high, regardless the conditions elsewhere: no differences in time until dispersal were found between the I and B treatment. These densities are comparable with maximal densities at the same food availability found in dos Santos, et al. (2008). At the time of their dispersal, inoculation plates had already reached higher population densities as in the D and N treatments, and intraspecific interactions may be increased. Organisms will disperse to avoid crowding, even though food is still available, in agreement with results on *C. elegans* (Harvey, 2009; Srinivasan, et al., 2012). In contrast with the clear density dependent effect in Pm I and Pm IV, Pm III dispersed before the fifth day in the B, I and D treatment, i.e. well before the first offspring generated in the inoculation plates became adult and density dependence could have become important. Pm II dispersed in all food treatments at a lower population density in the inoculation plate compared with the other species (but at the same time as Pm III and Pm IV). This can be the consequence of higher intraspecific competition in this species. The effect of food quantity was not tested in this experiment, but we can expect that lower food availability in the inoculation plates will result in more severe intraspecific competition behaviour. Previous results already showed an inverse relationship between food availabilities and dispersal rates in a variety of invertebrates and vertebrates (Kuussaari, et al., 1996; Lurz, et al., 1997; Oro, et al., 2004; Harvey, 2009).

**Salinity influenced time of dispersal**

Besides the species-specific effect of food distribution on dispersal, salinity also had an effect on dispersal, with a generally more rapid dispersal at the lower salinity for all four cryptic species. Despite this, no significant differences were found between the two salinities within individual species, even though the average in time until dispersal over the four cryptic species was 2.4 ± 1.0 days longer at the higher salinity. We had anticipated such a

response for Pm III and Pm IV, because monospecific cultures of both species had higher growth rates at the lower salinity (De Meester, et al., 2011; chapter II), so intraspecific competition could be expected to show up sooner at the lower salinity. However, both species showed significantly lower total densities in the inoculation plate at the time of dispersal at the lower salinity, demonstrating that the effect of salinity does not simply mirror density-dependence. We suggest that the salinity effect on dispersal may be a consequence of a different energy allocation at different salinities (Mole & Zera, 1993; Langellotto, et al., 2000; Zera & Harshman, 2001; Hughes, et al., 2003). When comparing total nematode densities of the present experiment with the results of a previous experiment without dispersal (De Meester, et al., 2011; chapter II), we see that Pm III reached higher total abundances at the lower salinity in cultures where no dispersal was possible compared with the present experiment in which dispersal was possible (resp. 132.8 ± 44.8 and 4.3 ± 2.6 nematodes at the time of dispersal, Kruskal-Wallis test, H= 10.59, P= 0.014; Fig. 5). These results should be interpreted with caution because both experiments were not performed simultaneously, but total population densities obtained in both experiments were comparable. These results support the energy allocation hypothesis: if organisms have the chance to disperse, they will spread their energy first over dispersal, and postpone reproduction until they arrive at the new plate, which is indicated by the rapid growth in the dispersal plate (around day 6 the population abundance in the dispersal plate was higher than in the inoculation plate in all 4 replicates; personal observation). These differences were not found at a salinity of 25, where Pm III showed comparable total densities in plates with and without dispersal opportunities (resp. 52.0 ± 16.9 and 10.5 ± 8.2 nematodes at the time of dispersal, ANOVA between two treatments, $F_{1,7}$=4.86, P= 0.07; Fig. 5). However, Pm III showed a higher juvenile density in the inoculation plate in the I treatment compared with the B treatment, which could be explained by the absence of a food trigger in the dispersal plate in the first treatment, leading to more investment of energy in reproduction than in dispersal. For Pm IV no differences were found between densities in plates with and without dispersal opportunities at lower salinity ($F_{1,6}$ = 0.07, P = 0.95; Fig. 5), so no differences in energy allocation were found for this species. The higher density in plates with dispersal opportunities at the salinity of 25 compared with the salinity of 15 could be due to differences in time until first dispersal, which was on average 2 days shorter at a salinity of 15 than of 25, although not significantly different from time until dispersal at the lower salinity (6.3 ± 0.9 days). These differences in densities were completely due to the number of juveniles ($F_{1,6}$ = 35.06 , P= 0.0010), which could point out that at the higher salinity the second

generation already started to reproduce in contrast with the population dynamics at the lower salinity. The increased population growth at a salinity of 15 in cultures without dispersal opportunities (De Meester, et al., 2011; chapter II) had no effect on the dispersal ability of the species and dispersal occurred at both salinities at a time when no differences in total densities between the two salinities were found (Fig. 5).

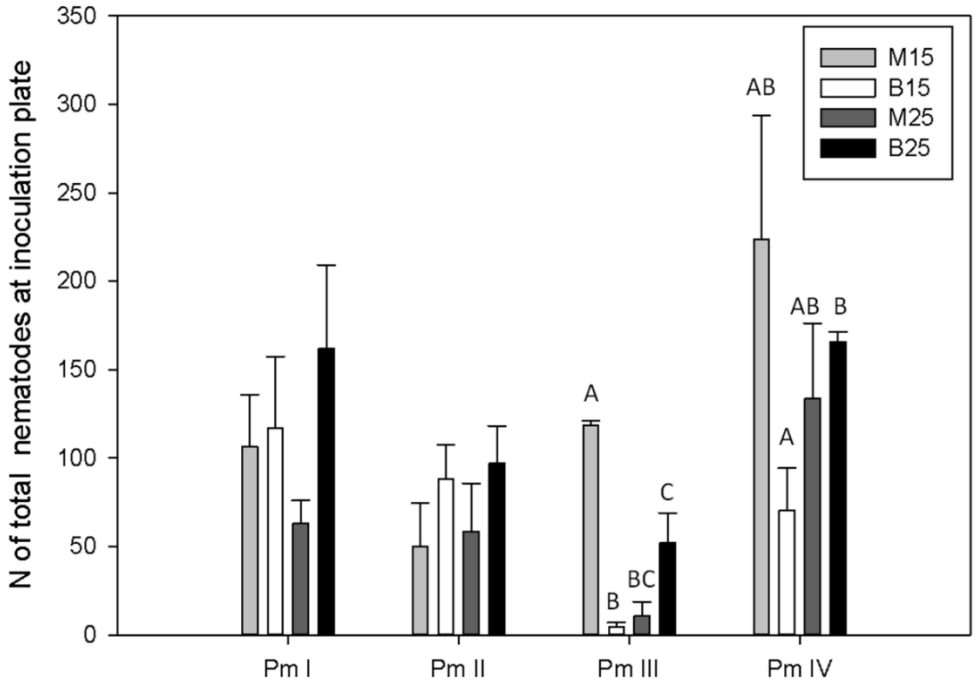



Figure 5: Average total number of organisms in the inoculation plate (mean ± SE) at time of first effective dispersal in plates with dispersal opportunities at two different salinities (B15:salinity of 15 ; B25: salinity of 25) compared with total number of organisms at the same time in plates without dispersal opportunities at the same two salinities (M15 and M25, data from De Meester, et al., 2011 (chapter II)) in four cryptic species of *L. "marina"* (letters above bars indicate significant differences; $p<0.05$; $n = 16$).

**Species-specific differences in number and gender of the dispersive organisms**

The number and gender of dispersive organisms also differed between the cryptic species. The salinity experiment showed that number of adults in the dispersal plate differed between Pm II and Pm III. Moreover, Pm IV followed the same trend as Pm II, and Pm I as Pm III. This trend was also found in the food distribution experiment. Pm I and Pm III had mostly only one or two dispersive individuals at the time of first dispersal and these were always females. In the days after the first dispersal event, males also arrived in the dispersal plate, invalidating the possibility of sex-biased dispersal in these species. The fact that in Pm I and Pm III the first dispersers were always females could theoretically be a consequence of female dominance in the populations. Preliminary results showed that for Pm III populations a biased male:female ratio exists (72.1 ± 11 % females), which could partly explain why females were the first dispersers, even though this sex ratio should not result in 100% of the first dispersers being females. Moreover, the male:female ratio is more balanced in populations of Pm I (53.8 ± 6.1 % females), so the fact that females were always the first dispersers clearly reflects sex-biased dispersal. This could result from fitness differences between males and females (Perrin & Goudet, 2001). Indirect support for this hypothesis comes from the observation that females of Pm I tend to have somewhat shorter development times than males (Moens & Vincx, 2000b; dos Santos, et al., 2008).The dispersal in the next days could then be triggered by the first dispersers, which leave mucus tracks on which bacteria can easily grow (Moens, et al., 2005), resulting in a food 'trail' towards the new patch. Pm II and Pm IV dispersed in most of the treatments with a higher number of organisms, with an almost perfectly balanced (1:1) ratio males:females. Here, it is more likely that individual rather than gender-specific differences in fitness (Clobert, et al., 2009) lead to specific dispersal abilities. Another possibility is that the species react differently to environmental cues or cues produced by conspecifics (Srinivasan, et al., 2012). When no food was available in both plates (N treatment), Pm IV dispersed with significantly fewer organisms compared with the D treatment, possibly the result of the absence of a food trigger. No such differences between food treatments nor between salinity treatments were, however, found in the other species, suggesting that the effects of environment on dispersal depend on the species.

**Highly efficient active dispersal within the cryptic species complex**

All four cryptic species showed highly efficient dispersal, the proportion of successful dispersal events exceeding 95% in all four cryptic species and under all experimental conditions (Kruskal-Wallis test for food, salinity and species: all $P>0.66$). The high dispersal rates observed here indicate that dispersal over short distances (10 cm) may be common in natural environments too. In natural environments dispersal will happen in a landscape mosaic (Wiens, 2001) and not just from one location to the other as in this experiment. Organisms will thus be able to move from and to different patches in search of better spots. This can lead to dispersal over larger distances. The fact that organisms only start to disperse after a few days instead of a few hours, can indicate that dispersal comes at a cost. Costs for active dispersal are mostly considered to be loss of reserves due to increased locomotory activity (Bonte, et al., 2011). Although these costs are expected to be small (only a few % of the total metabolic costs) , time and risk (for instance an increased predator-prey encounter probability by leaving more protected structures (such as floating bladders) to disperse) costs (Moens, et al., 2000) should also be taken into account. That dispersal goes with a cost is shown in the N treatment for Pm III, where the dispersal plate of one of the replicates went extinct. Moreover, time until dispersal was somewhat slower, which could be the result of the absence of food and thus energy resources. This trend was not seen in the other species. Dispersal can be a selective advantage when the fitness benefits of dispersal exceed the costs of movement (Bowler & Benton, 2005). When local conditions become less favourable (e.g. food depletion, higher intraspecific competition, etc.), dispersal will be beneficial.

**Differential dispersal abilities can help explain temporary coexistence**

Our study demonstrates that differences in time until dispersal between very closely related nematode species exist. Dispersal is in most cases density-dependent. However, Pm III had a shorter dispersal time compared with Pm I, and dispersed well before high densities were reached in the inoculation plate. Moreover, food distribution and salinity can alter the timing of dispersal in cryptic species of *L. "marina"*. This response is species- and condition-specific. If active dispersal is common in natural environments, patches where species go extinct, can easily become colonised again (Derycke, et al., 2007b), which can contribute to the resilience of populations (Harrison, 1979). The typical habitat of *L. "marina"* consists of ephemeral patches of macroalgal wrack washed ashore, and local populations are hence subject to pronounced colonization-extinction dynamics (Derycke, et al., 2007b). The species-specific differences in dispersal strategy can have important consequences for

metapopulation and metacommunity dynamics, genetic diversity and species composition in newly establishing populations and assemblages and can facilitate coexistence. If, for instance, priority effects - where the first arriving species will have an advantage over the following species - occur (Boileau, et al., 1992; Derycke, et al., 2007b), competitively inferior species may be able to coexist with more competitively superior species. Clear priority effects within a single cryptic species of *L. "marina"* (Pm I) have been demonstrated in a field experiment, impacting the genetic structure and diversity of local populations (Derycke, et al., 2007b). However, we are unaware of any studies demonstrating priority effects between different nematode species. The active dispersal observed here over small distances may affect dispersal at larger scales, since it may facilitate passive dispersal as well. The differences in dispersal can also affect the response of cryptic species to competition and can help explain temporary coexistence between cryptic species. For instance, weaker competitors could be expected to disperse sooner. From the mixed-species experiment by De Meester, et al. (2011; chapter II), however, Pm I and Pm III proved to be the stronger competitors and Pm II and Pm IV the weaker ones, so both the slowest and fastest disperser in our current experiments appear to be strong competitors. For testing this hypothesis, more information about the interaction between dispersal and other biological factors (e.g. competition) is necessary to better understand this coexistence. In a future experiment, microcosms with dispersal opportunities, in which all four cryptic species are placed together, will be started up to record differences in time until dispersal between the cryptic species when competition between the different species is present.

## Acknowledgements

Annelien Rigaux is acknowledged for help with the nematode counts. SD is a postdoctoral fellow of the Flemish Fund for Scientific Research FWO. Financial support for this study came from the special research fund (BOF) of Ghent University through projects B/09941and B/11644 and from the FWO through project B/07715. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

# 7

# CHAPTER VII

# DISPERSAL, ITS DRIVERS AND ITS IMPACT ON COMPETITIVE INTERACTIONS

---

Slightly modified from:

De Meester, N., Derycke, S., Rigaux, A. & Moens, T. (2015) Active dispersal is differentially affected by inter-and intraspecific competition in closely related nematode species. *Oikos,* 124, 561-570.

## Abstract

Competition is one of the main drivers of dispersal, which can be an important mechanism to achieve permanent or temporal coexistence of multiple species. This coexistence can be achieved by a competition-colonization trade-off, spatial storage or neutral dynamics. Here we test the effect of inter- and intraspecific competition on dispersal of four species of the marine nematode species complex *Litoditis "marina"*. A previous study in closed microcosms without a possibility for dispersal had demonstrated pronounced interspecific competition, leading to the exclusion of one species. We now investigated whether (a) the dispersal is affected by interspecific interactions, by intraspecific competition (density) or by food availability, (b) the dispersal dynamics influence assemblage composition and can lead to co-occurrence of the species, and (c) the abiotic environment (here salinity) can affect these dynamics. We show that density is the main driver for dispersal in two of the four species. Dispersal of a third species always started at the same time irrespective of density, whereas in the fourth species interspecific interactions accelerated dispersal. Remarkably, this fourth species was not a strong competitor, suggesting that a competition-colonization trade-off does not explain the observed coexistence. Salinity did not alter the timing of dispersal when interspecific interactions were present but did affect assemblage composition, with higher abundances of Pm III at the lower salinity compared with the higher salinity. Consequently, spatial storage may influence coexistence. All four species co-occurred in fairly stable abundances throughout the present experiment indicating the importance of species-specific dispersal strategies for coexistence. Co-occurrence can be facilitated because competition is postponed or avoided by dispersal. The neutral dynamics paradigm may also help to explain coexistence, as intra- and interspecific competition were of similar importance in three of the four species. We conclude that dispersal is a driver of the coexistence of closely related nematode species, and that population density and interspecific interactions shape these dynamics.

## Introduction

Dispersal, the movement away from the natal habitat, plays an important role in the dynamics and evolution of spatially structured populations (Ronce, 2007). Both intraspecific and interspecific competition are among the main processes shaping the evolution of dispersal, mostly through density-dependence, as interactions become stronger when density increases (Lambin, et al., 2001). According to classical competition theory, interspecific competition also increases with relatedness between species, rendering coexistence of closely related species unlikely (e.g. Violle, et al., 2011). Permanent coexistence of closely related species may exist if intraspecific competition is equal to or higher than interspecific competition, which can be the case if the different species are locally adapted to different resources (niche theory; Chesson, 2000a), or if neutral dynamics play a role. For identical competitors extinction-coexistence dynamics will be slow and random and dispersal may allow coexistence of highly similar species through neutral dynamics (Leibold & McPeek, 2006; Leibold, 2008). If interspecific competition is higher than intraspecific competition, dispersal can also be important to avoid competition (Waser, 1985). Dispersal can facilitate coexistence by allowing to build up population levels in favourable areas in which competing species will be spatially or temporarily segregated to some degree and only a temporary form of coexistence remains (Snyder & Chesson, 2003). We define such a temporary form of coexistence as co-occurrence (Leibold & McPeek, 2006).

Cryptic species are species that are morphologically indistinguishable, but show genetic differences. This cryptic diversity is important in terms of biodiversity estimates and ecosystem functioning (Zhang, et al., 2004; Bickford, et al., 2007). Several studies have shown that cryptic species co-occur at small geographical scales (Zhang, et al., 2004; Derycke, et al., 2006), which seems at odds with classical competition theory. The underlying mechanisms of this co-occurrence – and whether it is temporary (co-occurrence) or permanent (coexistence) – remain unknown.

Nematodes are the most abundant (densities between $10^5 – 10^8$ individuals $m^{-2}$) and species-rich meiofaunal group in marine sediments (Lambshead & Boucher, 2003). They may stimulate bacterial growth in microbial biofilm formation (Hubas, et al., 2010) and decomposition processes (Freckman, 1988), with closely related species sometimes exhibiting differential effects on bacterial growth, activity and diversity (De Mesel, et al., 2006; Hubas, et al., 2010). Moreover, these closely related species may also affect each other's population development (De Mesel, et al., 2006). Such local interactions, including

competition and facilitation, are likely to strongly influence nematode assemblage structure. However, such interactions have hitherto only been studied in 'homogeneous' microcosms, thereby ignoring the role of dispersal, which can lead to temporary forms of coexistence (Palmer, et al., 1996). In addition, the effect of intraspecific competition – i.e. competition for resources (e.g. food, mates and space) within a species – on the population dynamics and dispersal abilities of nematodes is also largely unknown.

Cryptic diversity is common in coastal nematodes (Derycke, et al., 2013). The best studied model 'species' in this context is *Litoditis "marina"* (henceforth referred to as *L. "marina"*, formerly known as *Rhabditis marina* or *Pellioditis marina*), which is mostly found associated with decomposing macroalgae. At least ten species can be found in this nematode morphospecies complex. These species seemingly lack distinctive morphological differences, but show consistent molecular divergences at nuclear and mitochondrial loci (COI, ITS, D2D3) (Fonseca, et al., 2008). Four of these species (Pm I, Pm II, Pm III and Pm IV) frequently co-occur in the littoral zone of the south-western coast and estuaries of The Netherlands (Derycke, et al., 2006; 2008b). Crossbreeding between the two most closely-related cryptic species (Pm I and Pm IV) does not occur (Fonseca, et al., 2008).

Information about niche differentiation among cryptic species of *L. "marina"* is still largely lacking. However, recent reports of differential population responses to temperature (De Meester, et al., 2015a; chapter III) and of differential resource utilization (Derycke, et al., 2016; chapter V) suggest at least some degree of niche differentiation. Moreover, the outcome of interspecific interactions between these cryptic species depended on salinity (De Meester, et al., 2011; chapter II), one of the key fluctuating factors in estuarine and coastal habitats. These interspecific interactions encompass competition and facilitation (De Meester, et al., 2011; chapter II). In closed, homogeneous microcosms, Pm I and Pm III were competitively superior and completely (Pm IV) or nearly (Pm II) excluded the other two species within ca. 8 generations. However, the experimental setup in De Meester, et al. (2011; chapter II) did not allow dispersal. Nematode dispersal is generally considered to be mostly passive (Palmer & Gust, 1985), but active dispersal over short distances also occurs (Ullberg & Ólafsson, 2003; Schratzberger, et al., 2004), and may facilitate active emergence from the sediment and in this way increase the chance of passive dispersal events over longer distances (Palmer, 1988). Differential active dispersal between the four cryptic species of *L. "marina"* has been demonstrated in a lab experiment (De Meester, et al., 2012; chapter VI): Pm III dispersed fastest while Pm I was the slowest disperser (Table 1). Salinity had an

influence on these dispersal abilities, with a generally faster dispersal at a lower salinity (15 compared to 25). Nematode density also affected the timing of dispersal, indicating that intraspecific competition can play a role in the dispersal behaviour. However, the effect of interspecific competition on dispersal in these cryptic nematode species has not been studied.

The purpose of this experiment was to assess whether (a) interspecific interactions, intraspecific competition and/or the effect of food availability are important triggers for dispersal in these nematode species, whether (b) dispersal will influence the assemblage composition in the inoculation and dispersal plates, and whether (c) external factors (here salinity) can shape population dynamics and affect the timing of dispersal.

Regional coexistence between the species can be achieved by competition-dispersal trade-offs, spatial storage (cfr. niche theory) or neutral dynamics (Amarasekare, 2003). If there is a trade-off between competition and dispersal, good dispersers will disperse before competition becomes too strong (McPeek & Holt, 1992). For the first purpose, we therefore expected that the weaker competitors (i.e. Pm II and/or Pm IV, see Table 1) will be the first dispersers (Cadotte, 2007). In addition, density-dependent dispersal exists in Pm I and Pm IV (De Meester, et al., 2012; chapter VI), so we expected intraspecific competition to be an important cause of dispersal in these two species. If interspecific and intraspecific competition are equally important, we can expect that differences between the species are minimal and neutral dynamics play an important role. The effect of food availability as a driver of dispersal in nematodes is still largely unknown. In a previous monospecific dispersal experiment (De Meester, et al., 2012; chapter VI) the absence of food caused faster dispersal, so we can expect that lower food availability per capita leads to faster dispersal. For the second purpose, we expected that dispersal would at least partly alleviate interspecific competition, thus facilitating co-occurrence. For the third purpose, more and faster dispersal and more differences in population dynamics between the species are expected at a lower salinity, due to the faster dispersal of all species (De Meester, et al., 2012; chapter VI) and the stronger competition observed at lower salinity (De Meester, et al., 2011; chapter II). Differential responses of the species to abiotic conditions could indicate spatial storage leading to coexistence.

Table 1: Overview of the most important differences in effects of salinity, competition and dispersal between the different cryptic species of *Litoditis "marina"*: A) data from De Meester, et al. (2011 (chapter II), 2012 (chapter VI)), B) data from De Meester, et al. (2011; chapter II) and the present experiment and C) conclusions of the present experiment.

| | | Pm I | Pm II | Pm III | Pm IV |
|---|---|---|---|---|---|
| A) Without interspecific interactions | **Effect of salinity on population abundance (without dispersal)** | No | No | Yes | Yes |
| | **Dispersal ability** | Low | Moderate | Moderate-High | Moderate |
| B) With interspecific interactions | **Competitive abilities without dispersal** | Superior | Inferior | Superior | Inferior |
| | **Dispersal ability** | Moderate | Moderate | Moderate | Moderate |
| | Main driver of dispersal (C) | Interspec. Comp. | Time-dependent | Density-dependent | Density-dependent |

## Material & Methods

### Nematode cultures

Nematodes for our experiments were harvested from monospecific stock cultures in exponential growth phase. Monospecific cultures of the four different cryptic species were raised from a single gravid female per species and maintained on sloppy (1%) nutrient:bacto agar media (temperature of 20°C; salinity of 25) with unidentified bacteria from their habitat as food (Moens & Vincx, 1998).

### Interspecific competition treatment (IC)

To study the effect of interspecific competition on the time until dispersal, the consequences of dispersal on the assemblage dynamics and the effect of salinity on both specially designed dispersal plates were used. These consisted of two Petri dishes (resp. 'inoculation' and 'dispersal' plate, 5 cm inner diameter) connected by a tube (1 cm inner diameter and 10 cm length) (De Meester, et al., 2012; chapter VI). Bacto agar medium (1.5%, 60 mL, prepared with artificial seawater was provided as a substratum (De Meester, et al., 2012; chapter VI). All plates contained agar with a salinity of 15 or 25 (i.e. two salinity treatments). The pH of the agar medium was buffered at 7.5 – 8 with TRIS-HCl in a final concentration of 5mM. Cholesterol (100 µL $L^{-1}$) was added as a source of sterols. Food consisted of frozen-and-thawed *Escherichia coli* (strain K12, 200 µL of a suspension with a density of $3x10^9$ dead cells $mL^{-1}$ (dos Santos, et al., 2008)) and was added every eighth day to both plates. No food was ever added to the connection tube.

An artificial assemblage composed of equal abundances of the four cryptic species was placed at the inoculation plate, and both the species identity of the first disperser and the assemblage composition of the inoculation and dispersal plates at different time moments were investigated at two different salinities (same salinities (15 and 25) as in De Meester, et al. 2011 (chapter II), 2012 (chapter VI)). The experiment was started by manually picking up five adult males and five adult females per species from the stock cultures and transferring them to the inoculation plate, yielding a total of 40 nematodes per inoculation plate. Before placing the organisms randomly in the inoculation plate, they were bathed in sterile artificial seawater (salinity of 25) for 2 h to remove most adhering bacteria.

Five independent sets of dispersal plates were made. The first set was used for measuring time until first dispersal, the others for analysis of assemblage dynamics at four moments in time, i.e. 5, 10, 15 and 20 days after inoculation. Every set consisted of four replicates each

for the two salinity treatments. All plates were incubated in the dark at a constant temperature of 20°C.

*A. Time until dispersal*

Dispersal abilities of the cryptic species were measured as time until the first dispersal event. The timing of the arrival of the first organism at the dispersal plate was recorded, as well as the life stage (adult or juvenile) and the number of adults and juveniles in the inoculation plate at time of dispersal, by checking the plates daily for dispersers. Organisms arriving at the dispersal plate were manually picked up for species identification using qPCR (see below). The experiment was ended on average two days after the first dispersal event, when too many eggs and juveniles were usually present on the dispersal plate and it became impossible to distinguish juveniles that had moved to the dispersal plate from those just hatched there. These results were compared with the results of the monospecific experiments (see below), to elucidate the effect of interspecific and intraspecific competition on the dispersal abilities of the species.

*B. Assemblage composition*

Assemblage dynamics were studied by counting adults and juveniles in both plates on a daily basis. Four dispersal plates per treatment and time (one set) were frozen at -20°C for species identification with qPCR analysis after each of the following incubation times: 5, 10, 15 and 20 days.

*C. Identification of nematodes*

To measure the time until first dispersal, we identified the nematodes which had reached the dispersal plate using qPCR analysis. Nematodes were handpicked one by one and transferred to a 0.5 ml Eppendorf tube containing 20 µL lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % NP 40, 0.45 % Tween20). Tubes were frozen at –20 °C, after which 1 µl of proteinase K (10 mg $ml^{-1}$) was added. Lysis took place in an Eppendorf Mastercycler gradient PCR machine (65 °C for 1 h, 10 min at 95 °C) and was followed by centrifugation of the DNA samples for 1 min at 14000 rpm (Derycke, et al., 2012).

Identifications of the cryptic species were performed with qPCR (Derycke, et al. 2012) using a Lightcycler 480 System (Roche). The qPCR mixture was prepared for a 10 µL reaction volume on 384-well plates using 5 µL SensiMix SYBR No-ROX One-Step (2x) solution (Bioline), 3 µL of each primer (species-specific primers were developed in the ribosomal internal transcribed spacer region (ITS) and were used in final concentrations of 1 µM for

Pm I and Pm III, 500 nM for Pm II and 200 nM for Pm IV (Derycke, et al. 2012)), 1 µL PCR-grade water and 1 µL of DNA template. The thermal cycling protocol consisted of an initial denaturation for 10 min at 95°C followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 20 s at 60°C, and extension for 20 s at 72°C.

For the analysis of the assemblage composition contents of the inoculation and dispersal plates were frozen separately (- 20°C) and later melted in hot distilled water (70°C). After complete melting of the agar, it was sieved into two different size sections to separate adults from juveniles using sieves with mesh sizes of 125 µm and 32 µm. A 95 % separation of adults and juveniles is achieved by this method. Subsequently, DNA was prepared from either size fraction, using hexadecyltrimethylammonium-bromide (CTAB) (protocol as described in Derycke, et al. 2012). The amount of DNA was measured with a Nanodrop 2000 (Isogen Life Science) and concentrations above 10ng $\mu L^{-1}$ were diluted, because excessive concentrations of DNA can interfere with the quality of the qPCR (Derycke, et al. 2012). The same protocol for qPCR as described above was used. Relative quantification was used for the assessment of the assemblage composition: differences in Ct values were calculated using the adjusted ΔΔCT method (Derycke, et al., 2012).

**Monospecific experiment (M-BF, M-HF and M-HD treatments)**

To elucidate the effects of inter- and intraspecific interactions and food availability on nematode dispersal, the results of the interspecific competition experiment (IC) were compared with those of three monospecific dispersal treatments. The "monospecific – basal food condition" treatment (M-BF) consisted of 10 organisms (five males and five females) of one species and 50 µL of $3x10^9$ *E. coli* cells $mL^{-1}$. In this treatment the total amount of food was 4 times lower than in the IC treatment, but the amount of food per inoculated organism was the same in both. This treatment was the same as in De Meester, et al. (2012; chapter VI). The "monospecific – high food condition" treatment (M-HF) also consisted of 10 organisms of one species, but here 200 µL of $3x10^9$ *E. coli* cells $mL^{-1}$ was added, identical to the total amount of food offered in the IC treatment. In the "monospecific – high density" treatment (M-HD), 40 organisms of one species and 200 µL of $3x10^9$ *E. coli* cells $mL^{-1}$ were added. Each of the three monospecific treatments was performed with each of the four cryptic species of *L. "marina"*. In this way we were able to investigate (1) the relative importance of interspecific vs. intraspecific competition as drivers of dispersal (IC treatment compared with M-HD treatment), (2) the importance of food availability (M-BF compared with M-HF), and (3) the role of the inoculum density (M-HF compared with M-HD

treatment). All conditions were the same as in the interspecific competition experiment, but only the higher salinity (25) was used. Time until dispersal was measured in the same way as in the interspecific competition experiment. An overview of all these treatments is found in Table 2.

Table 2: Set-up of the combination of the monospecific experiment and the interspecific interaction experiment: A) design of the different treatments with their codes and B) rationale and aims of the different treatment comparisons.

| | | |
|---|---|---|
| A) | M-BF (monospecific – basic food): | 10 nematodes, 1 species, 50µL food |
| | M-HF (monospecific – high food): | 10 nematodes, 1 species, 200 µL food |
| | M-HD (monospecific – high density): | 40 nematodes, 1 species, 200 µL food |
| | IC (interspecific competition): | 40 nematodes, 4 species, 200 µL food |
| B) | IC vs. M – HD : | Interspecific vs. intraspecific competition |
| | M-BF vs. M-HF: | Effect of food availability |
| | M-HF vs. M-HD: | Effect of inoculum density (density-dependence) |

**Statistical analyses**

For the interspecific competition experiment, the effects of salinity and species identity on time until dispersal were investigated in PRIMER (Clarke & Gorley, 2006) using a Permutational Based Multivariate Analysis of Variance on the basis of Euclidean distance with 999 permutations (PERMANOVA (Anderson, 2001)), as data were not normally distributed, even after transformation. Differences in densities of adults and juveniles in the inoculation plate at time of first dispersal event (regardless which species dispersed first) between the salinities were tested with a one-way ANOVA in R (R Development Core Team, 2008). For adult density a log-transformation was needed to normalise the data.

PERMANOVA was also used to investigate the adult and juvenile assemblage composition of both dispersal and inoculation plates in the interspecific competition experiment. The relative contribution of every species was the dependent variable. Time (excluding the T0 species composition), plate (inoculation or dispersal plate) and salinity (15 or 25) were the independent fixed factors. Significant terms and interactions were investigated using posterior pair wise comparisons within PERMANOVA. A SIMPER analysis was used to

identify which species primarily accounted for the observed differences. PERMDISP was performed to test the homogeneity of multivariate dispersions (distance to the centroid). Log-transformation was performed for the adult assemblage composition to achieve this homogeneity.

For the monospecific experiment, time until dispersal and adult and juvenile densities at time of first dispersal were compared between the different treatments (M-BF, M-HF, M-HD) and with the interspecific interactions experiment (IC). PERMANOVA (on the basis of Euclidean distance with 999 permutations) was used to investigate the effect of species and treatment on time until dispersal, because data were not normally distributed. Two-way ANOVA in R was used for the adult and juvenile densities in the inoculation plate at first dispersal event (log-transformation for juvenile data). Pairwise PERMANOVA tests and Tukey Honest Significant Differences test were respectively conducted to investigate pairwise differences of the highest level significant factor(s). The results of these tests were used to answer our research questions (Table 2b). To investigate the effect of the treatments in more detail, one-way ANOVAs were used for each species separately. A Kruskal-Wallis test was used for the juvenile density at first dispersal event for Pm IV as a non-parametric alternative because the data did not fit the requirements for parametric tests, even after transformation.

## Results

### Effect of inter- and intraspecific interactions on the dispersal of the *L. "marina"* species

All four cryptic species dispersed at the same time in the IC treatment, irrespective of salinity (Fig. 1, Table 3). However, when comparing the monospecific experiments with the interspecific competition experiment, species identity did significantly influence time until dispersal as well as adult and juvenile density at time of first dispersal (Table 4). Regardless treatment (M-BF, M-HF, M-HD or IC), Pm I was the slowest disperser and dispersed 2.9 ± 1.9 days later than Pm IV. Higher overall adult densities of Pm III in the inoculation plate at time of first dispersal occurred compared with the other three species (78.3 ± 28.8 adults compared with 18.1 ± 3.3 (Pm I), 20.2 ± 10.0 (Pm II) and 7.9 ± 2.3 (Pm IV)). Moreover, considerably higher densities of juveniles of Pm III (413.3 ± 163.3) at first dispersal event were also found compared to Pm II and Pm IV (resp. 52.3 ± 6.5 and 43.7 ± 9.9) (data not shown).

Table 3: Results of the two-way ANOVA of the interspecific competition experiment (IC treatment), independent factors: salinity (15 and 25) and species identity (Pm I, Pm II, Pm III or Pm IV) on time until dispersal. Level of confidence = 95 %.

| Effect on time until dispersal | Df | $F_{3,24}$ | p |
|---|---|---|---|
| **Species identity** | 3 | 2.97 | 0.064 |
| **Salinity** | 1 | 1.10 | 0.29 |
| **Salinity*Species identity** | 3 | 1.55 | 0.26 |

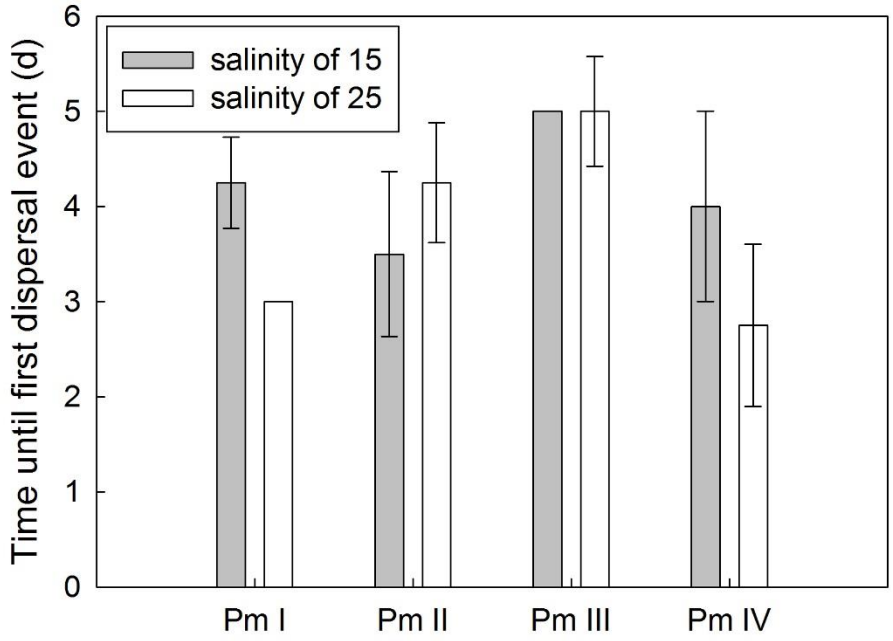



Figure 1: Average time until first dispersal event (mean ± SE) for the four cryptic species of *Litoditis "marina"* at two different salinity treatments (resp. 15 and 25) (n = 32) in the interspecific competition experiment.

Time until dispersal was influenced by the treatments (M-BF, M-HF, M-HD or IC) with later dispersal of all species in the treatment with low inoculum density and high food availability (M-HF treatment) compared with the higher inoculum density and the same food availability (M-HD treatment) (resp. after 7.3 ± 0.6 days and 4.9 ± 1.2 days), but not significantly influencing the adult and juvenile densities at time of first dispersal (Table 4).

Within-species analysis revealed more information on the effect of the treatments on time until dispersal and on adult and juvenile densities at the inoculation plate at time of first dispersal (Fig. 2). For Pm I, there was only an effect on time until dispersal ($F_{3,12}$=4.86 , p=0.019), with Pm I nematodes dispersing after less than half the time in the presence of other species (IC) compared to dispersal in most of the monospecific treatments ($p < 0.05$ for IC with M-BF and with M-HF) (Fig. 2). No significant differences were found between IC and M-HD (interspecific vs intraspecific competition), M-BF and M-HF (effect of food availability), or M-HF and M-HD (density-dependence). For Pm II, adult numbers at the inoculation plate at time of first dispersal were significantly higher in the M-HD treatment than in the three other treatments ($F_{3,12}$=8.81, p=0.0023), showing that Pm II dispersed at higher adult densities when there were no other species present and when the inoculum

density was higher (Fig. 2). For Pm III, time until dispersal and adult and juvenile numbers at the inoculation plate were not influenced by the treatments (M-BF, M-HF, M-HD or IC) ($F_{3,12}$, all $p>0.05$; Fig. 2). Pm IV had a slower dispersal in the M-HF treatment compared to the M-HD and IC treatments, as also seen in the main effect above. Moreover, adult densities at time of first dispersal were significantly higher in the IC than in the M-BF treatment, but no difference between IC and M-HD, M-BF and M-HF and M-HF and M-HD occurred (Fig. 2).

Table 4: Results of the two-way ANOVA of the monospecific and interspecific interactions experiment (independent factors: treatment (M-BF, M-HF, M-HD or IC) and species identity (Pm I, Pm II, Pm III or Pm IV)) on time until dispersal, adult and juvenile density at time of first dispersal event. The p-values of the pair-wise differences can be found for each significant factor (results of Tukey Honest Significant Differences Test). Level of confidence = 95 %.

| | **Df** | **Time until dispersal** | | **Adult density** | | **Juvenile density** | |
|---|---|---|---|---|---|---|---|
| | | F | P | F | P | F | P |
| **Species** | 3 | 4.31 | **0.013** | 4.57 | **0.01** | 6.94 | **< 0.0001** |
| Pm I – Pm II | | | 0.16 | | 0.99 | | 0.99 |
| Pm I – Pm III | | | 0.21 | | **0.031** | | 0.058 |
| Pm I – Pm IV | | | **0.004** | | 0.96 | | 0.99 |
| Pm II – Pm III | | | 0.99 | | **0.041** | | **0.036** |
| Pm II – Pm IV | | | 0.48 | | 0.94 | | 0.99 |
| Pm III – Pm IV | | | 0.39 | | **0.0087** | | **0.031** |
| **Treatment (M-BF, M-HF, M-HD or IC)** | 3 | 8.61 | **0.001** | 1.84 | 0.15 | 1.75 | 0.17 |
| IC – M-HD | | | 0.53 | | | | |
| M-BF – M-HF | | | 0.99 | | | | |
| M-HF – M -HD | | | **0.048** | | | | |
| **Species*Treatment** | 9 | 1.58 | 0.14 | 0.80 | 0.62 | 1.39 | 0.22 |

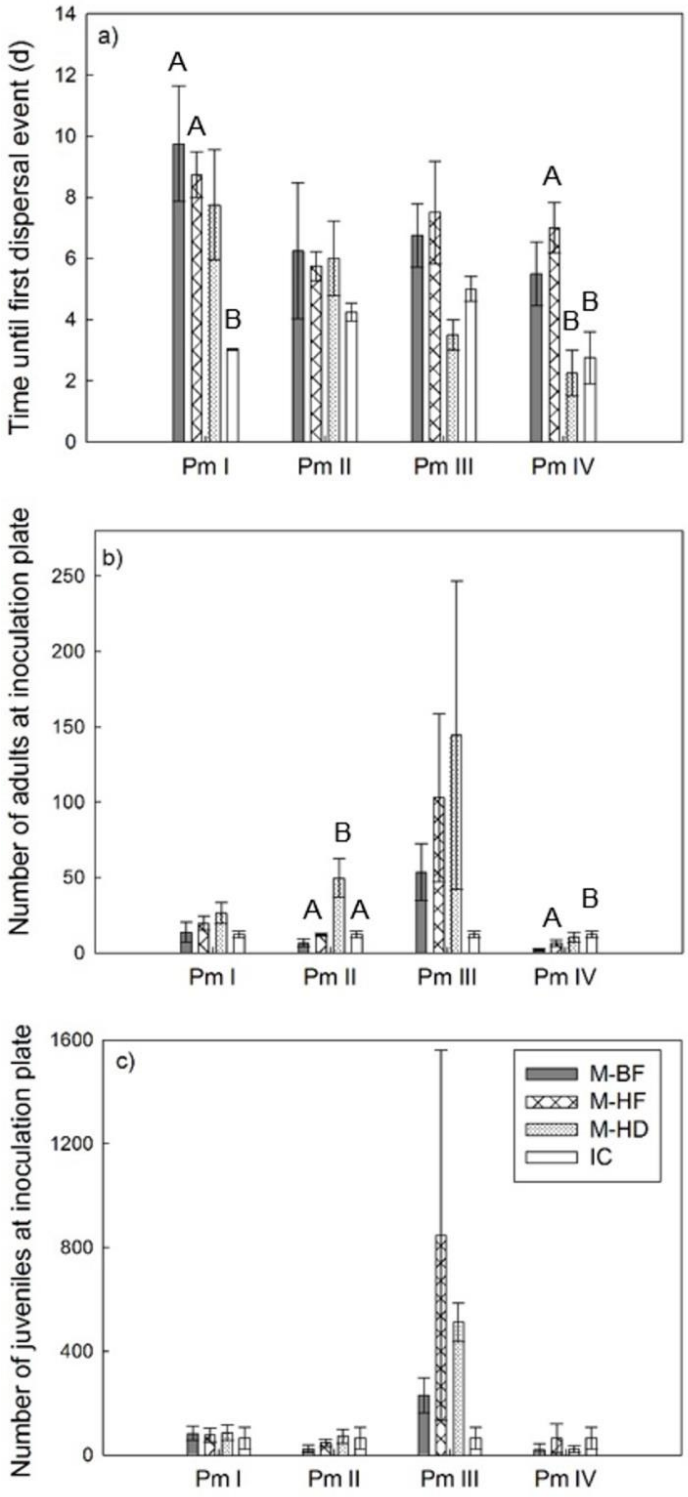



Figure 2: Effect of treatments (M-BF, M-HF, M-HD and IC) on (a) time until first dispersal, (b) number of adults and (c) number of juveniles of *Litoditis "marina"* in the inoculation plate at time of first dispersal event (mean ± SE). For the IC treatment, numbers of adults and juveniles at time of first dispersal are regardless species identity and as a consequence these values are identical for all the species. Letters indicate pairwise significant differences within species ($p<0.05$, $n=64$).

**Consequences of dispersal on assemblage dynamics**

All four cryptic species were present in both plates at days 5, 10, 15 and 20. At the end of the experiment (after 20 days), all species were still present in more or less the same relative abundances in both plates (Fig. 3). Both adult and juvenile assemblage compositions did not differ significantly between the different time moments or between the different plates (inoculation versus dispersal plate). Salinity did have an effect on the adult, but not on the juvenile assemblages (Fig. 3) (see below). No interaction effects between plates, salinity and time were found (Table 5).

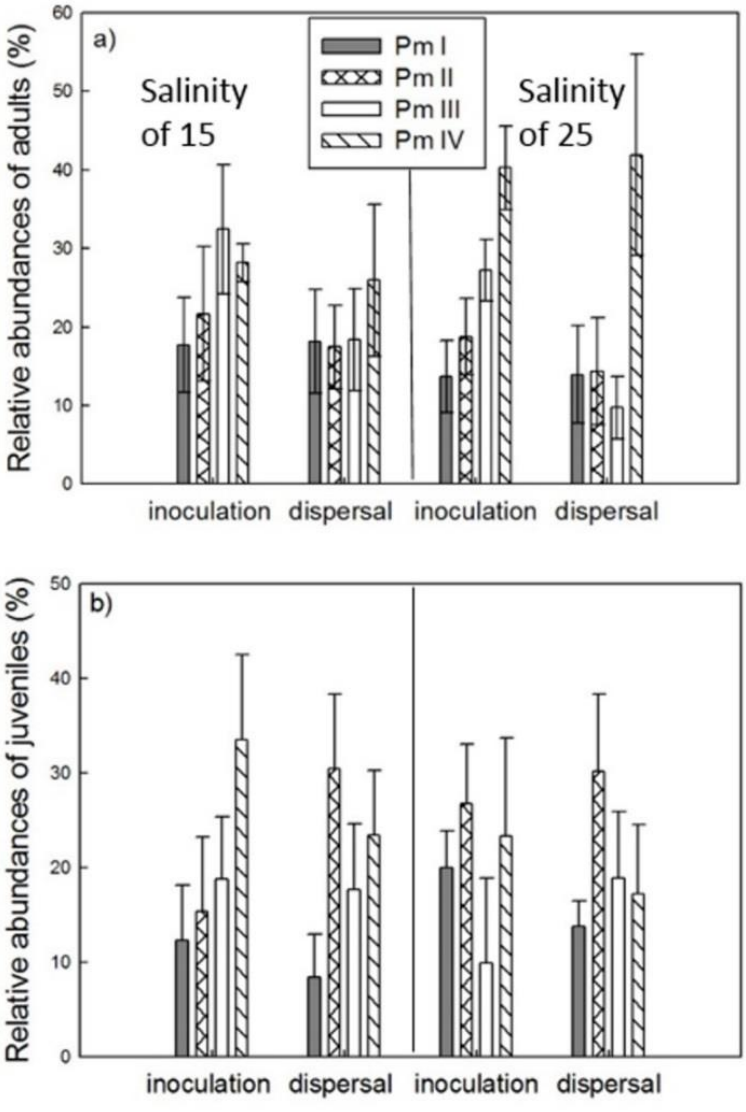



Figure 3: Relative abundances of adults (a) and juveniles (b) (average % ± SE) of the cryptic species complex of *Litoditis "marina"* (IC experiment) at 20 days (end of the experiment) for the inoculation and dispersal plates at the two different salinities.

Table 5: Results of the PERMANOVA on the assemblage composition of adults and juveniles (independent factors: salinity (15 or 25), time (5, 10, 15 or 20 days) and plate (inoculation or dispersal plate). Level of confidence = 95 %.

| | | Adults | | Juveniles | |
|---|---|---|---|---|---|
| | **Df** | **Pseudo-F** | **P** | **Pseudo-F** | **P** |
| **Salinity** | 1 | 3.53 | **0.009** | 0.57 | 0.64 |
| **Time** | 3 | 1.13 | 0.34 | 1.21 | 0.29 |
| **Plate** | 1 | 0.75 | 0.57 | 0.83 | 0.45 |
| **Salinity*time** | 3 | 1.49 | 0.15 | 0.86 | 0.55 |
| **Plate*time** | 3 | 1.70 | 0.073 | 0.70 | 0.75 |
| **Salinity*plate** | 1 | 1.81 | 0.13 | 0.86 | 0.46 |
| **Salinity*plate*time** | 3 | 1.47 | 0.13 | 0.58 | 0.82 |

**Effect of salinity on dispersal and competition**

In the interspecific competition experiment, no effect of salinity was found on time until dispersal (see Table 3). Total nematode density, covering all four cryptic species together, at time of first dispersal, differed between the salinities, with higher densities at lower salinity. This result was only significant for the adults ($F_{1,6}$=0.42, p=0.040, Fig. 4), with more than twice the number of adults at time of first dispersal at the lower compared to the higher salinity.

Differences in adult assemblage composition were observed between different salinities (Table 5), primarily due to Pm III (32.92%) having higher relative abundances at the lower salinity, compared with the higher salinity (Fig. 3),. No effects of salinity on the juvenile assemblage composition were found (Table 5).

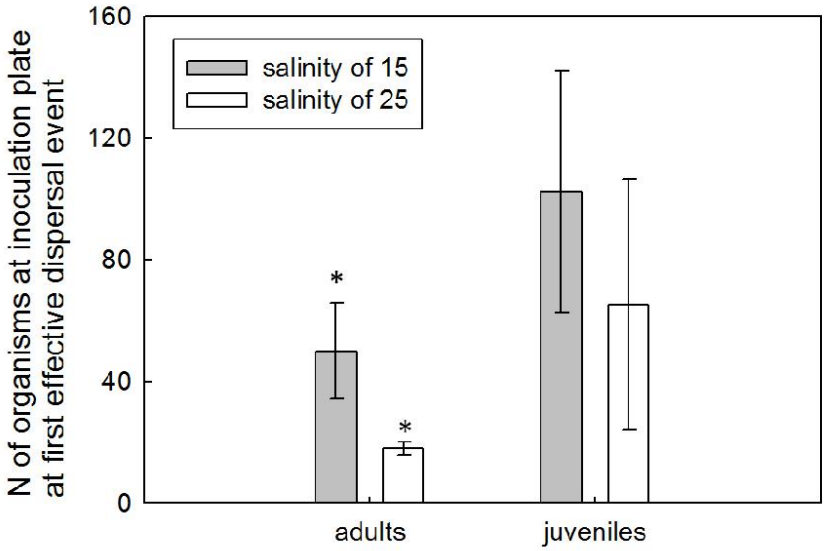



Figure 4: Average numbers of adults and juveniles (mean ± SE) in the inoculation plate at time of first dispersal (regardless species identity) of *Litoditis "marina"* at two different salinity treatments (resp. 15 and 25) (* indicates significant pairwise differences; $p < 0.05$ ; n=8).

## Discussion

Dispersal may be an important mechanism to achieve co-occurrence of closely related and highly similar species through a temporary avoidance of high interspecific competition. The co-occurrence of closely related species in patchy environments despite competition can be explained by (1) competition-dispersal trade-offs, (2) spatial storage (cfr. niche theory) or (3) neutral theory (Amarasekare, 2003). Our results (combined with the results of De Meester, et al., 2011 (chapter II), 2012 (chapter VI)) show that at least two explanations (option 2 and 3) can apply to the *Litoditis "marina"* cryptic species complex. An overview of the results can be found in Table 1.

**Intra- and interspecific interactions strongly affect dispersal in a species-specific way**

A generally faster dispersal was found in the treatments with a higher inoculum density of nematodes (M-HF differed from M-HD: results of the two-way ANOVA (tabel 4)). Density was the main driver of dispersal, regardless species, however, the adult and juvenile densities in the inoculation plate at time of first dispersal did not differ between the treatments, indicating that the faster dispersal in the treatments with larger inoculum size was mainly due to a faster population growth, as dispersal occurred at the same densities as in the treatment with lower inoculum density. Such a density-dependent dispersal suggests that organisms will only disperse when a certain density of nematodes is reached, and suggests that the cost of dispersal is relatively high and that individuals tend to remain in a patch until competition exceeds a certain threshold (Travis, et al., 1999).

For Pm IV this density-dependent dispersal was confirmed in the one-way ANOVA within species: Pm IV dispersed faster at a higher inoculation density (M-HD) compared with the lower inoculum density (M-HF). Nevertheless, dispersal always occurred at the same nematode density, irrespective of the presence of competing species, which suggests the existence of a 'threshold density', i.e. a density at which interactions between organisms and/or species become too strong, and which triggers organisms to disperse. Competition for food will be unlikely because species did not disperse earlier at lower food conditions (M-BF), so competition for space or other resources or interference competition can be the main triggers. Such a threshold density for dispersal has already been observed in a wide range of terrestrial organisms (e.g. Doncaster, et al., 1997; Travis, et al., 1999) and in some freshwater and marine invertebrates (Service & Bell, 1987; Commito, et al., 1995; Fonseca & Hart, 1996). This result also implies that for Pm IV, interspecific and intraspecific interactions were equally important triggers of dispersal (Fig. 2; no differences found between M-HD

and IC treatment). This is surprising given that this species was competitively excluded by the other species in the absence of dispersal possibilities (De Meester, et al., 2011; chapter II), so we expected that interspecific interactions would be an important trigger for dispersal. The result in the present experiment indicates that intraspecific competition may also be one of the drivers, next to interspecific competition, of the exclusion of this species in the competition experiment of De Meester, et al. (2011; chapter II). In general, caution is due when extrapolating our results to field conditions, since our lab experiments did not allow variations in food quality and abiotic conditions (others than salinity), which may have an important differential influence on interspecific and intraspecific interactions, as shown in plant experiments (Light, et al., 1983).

Density-dependent dispersal also occurred in Pm III: dispersal in this species always occurred at the same time, but Pm III population abundances increased more slowly at a higher inoculum density; in this way, dispersal always occurred not only at the same time, but also at the same nematode density. This threshold density was higher for Pm III than for the other species, which may be one of the reasons why Pm III was dominant in a closed multispecies experiment (De Meester, et al., 2011; chapter II): competition (intra- and/or interspecific) for other species became too high when nematode densities increased, but for Pm III these densities where still low enough to allow further population growth. Support for the hypothesis of a higher threshold for dispersal in Pm III also comes from the observation that monospecific cultures of this species at 20°C and a salinity of 25 maintained a high growth rate for a longer period than cultures of the other species, resulting in maximal population densities that were five times higher than in the other species (De Meester, et al., 2015a; chapter III).

In contrast to Pm III and Pm IV, Pm I and Pm II did not show a clear density-dependent dispersal (non-significant results in within-species ANOVA for Pm I and Pm II). Pm II always dispersed at the same time, regardless treatment, but the density in the inoculation plate at first dispersal differed between the treatments, suggesting intraspecific competition not to be the prime trigger of dispersal. This result could indicate that the cost to disperse is lower for Pm II than for the other species. When considering all treatments, Pm I was the slowest disperser, in accordance with De Meester, et al. (2012; chapter VI). However, this species dispersed equally fast as the other species when interspecific interactions occurred (Fig. 1) suggesting that interspecific interactions accelerate the timing of dispersal in Pm I. This faster dispersal of Pm I is unlikely to be the result of a competition- colonization trade-

off (Cadotte, 2007): better colonizers (better dispersers and higher reproductive output) would then be predicted to be poorer competitors and vice versa. However, Pm I is a strong competitor (De Meester, et al., 2011; chapter II) and was the slowest disperser in the monospecific experiment (De Meester, et al. 2012 (chapter VI) and the current experiment (the M-BF treatment is similar to the experiment of 2012 and no significant differences were found between these two experiments)), but it nevertheless dispersed faster when other species were present. This dispersal can indicate that the cost of dispersal is lower than the cost to compete (Waser, 1985; Gandon, 1999). Alternatively, the dispersal of Pm I could have been triggered by the first dispersers of another species, which leave mucus tracks on which bacteria can grow, resulting in a food 'trail' towards the new patch (Moens, et al., 2005), thus facilitating dispersal. However, total densities (over all four species) in the inoculation plate at time of first dispersal were not significantly lower in the IC treatment than the Pm I densities in other treatments, suggesting density-dependence still plays a role. Hence, both intra- and interspecific competition may be important drivers of dispersal in Pm I.

The present experiment also clearly shows that food availability is not a major driver of dispersal, which may be due to the small range in food availabilities used in our treatments. Larger differences in food availability have been shown to profoundly affect population growth rates of Pm I (dos Santos, et al., 2008) and could thus indirectly have an effect on dispersal. Other possible reasons to disperse at high population density are space limitation and/or interference competition (Hastings, 1980).

**Dispersal resulted in co-occurrence of the four cryptic species**

Our results show that dispersal can result in at least a temporary form of coexistence between closely related species (which has aslo been found in parasitoid invertebrates, Hassell, et al., 1994). All species had comparable abundances, both in the inoculation and dispersal plates, from day 10 onwards, and no exclusion of species occurred during the whole duration of the experiment. In a previous experiment with the same starting densities but without dispersal opportunities (De Meester, et al., 2011; chapter II) and a duration of 35 days Pm IV completely disappeared from the adult population and Pm II went extinct at a salinity of 15 but remained present at a salinity of 25, albeit in low abundances. Only data of adults were analysed in that experiment, so it is possible that juveniles remained present. In contrast, all species were able to persist as adults and juveniles throughout the present experiment, which could be partly due to the shorter duration of the present experiment (only 20 days, due to

the limited size of the dispersal plates). However, in the experiment by De Meester, et al. (2011; chapter II), exclusion of Pm IV at a salinity of 15 had already occurred after 15 days, and population abundance had also strongly dropped by that time at a salinity of 25. A PERMANOVA analysis on the time-averaged data of the adults from both experiments showed that the assemblage compositions differed between the inoculation and dispersal plates of the dispersal treatments on the one hand, and the plates without dispersal opportunities on the other (data from De Meester, et al. 2011; chapter II) ($F_{2,23}$=4.11, p=0.004, Fig. 5). These results should be interpreted with caution because both experiments were not performed simultaneously or under the exact same conditions. Nevertheless, total population densities obtained in both experiments were comparable. Moreover, the densities at which dispersal occurred in the current experiment were comparable to the densities at which the effects of interspecific interactions became clear in the competition experiment (De Meester, et al., 2011; chapter II). In addition, monospecific cultures without dispersal reached similar maximum abundances (De Meester, et al., 2011; chapter II) as in the present experiment, leading to the conclusion that intraspecific competition was also present in this former experiment.

Our results show that dispersal and competitive interactions (interspecific and intraspecific) influence each other. Escaping conspecifics or individuals of closely related species when densities become too high is a major potential benefit of dispersal (Van Valen, 1971). Co-occurrence of the closely related species is facilitated by dispersal to patches with lower abundances because competition can be postponed or avoided (Hanski, 1999; Leibold, et al., 2004). It is plausible that competition would have occurred had the experiment been maintained longer, and population abundances would have further increased on both inoculation and dispersal plates. In a natural environment with a patch-dynamic architecture, organisms can move to more suitable patches when interspecific or intraspecific interactions become too strong (Cohen & Levin, 1991), which is part of the metacommunity theory (Leibold, et al., 2004). In natural environments, abundances of the four cryptic species of *L. "marina"* differ at a regional scale and between seasons (Derycke, et al., 2006). Dispersal can be one of the factors influencing the different compositions at different moments in time and leading to co-occurrence at local, and to coexistence at regional scales (Amarasekare, 2003). The active dispersal observed here is likely more prominent at local scales, whereas passive dispersal, alone or in combination with active dispersal, is expected to predominate at larger scales (Ullberg & Ólafsson, 2003).

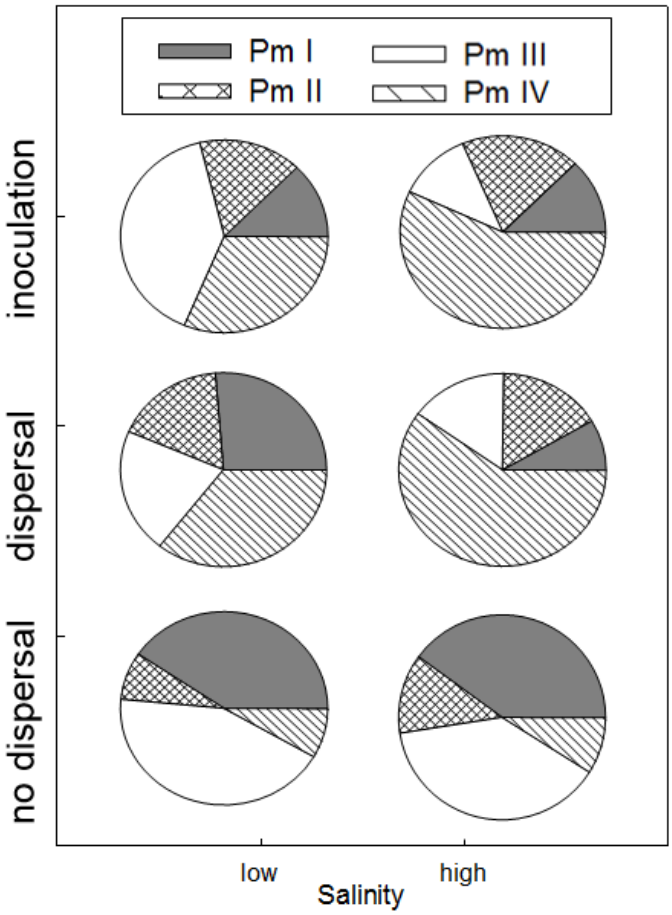



Figure 5: Time-averaged assemblage compositions (relative abundances of the four cryptic species of *Litoditis "marina"*) in the current experiment ('inoculation' and 'dispersal' for resp. both plates of the dispersal plate) and the experiment of De Meester, et al. (2011 (chapter II), no dispersal treatment) at two different salinities (15 and 25) (n=24).

**Salinity did not alter time until dispersal**

Abiotic factors can affect the interactions between species and their dispersal abilities (e.g. Walters & Bell, 1986; Fournier & Boivin, 2000) and can thus play an important role in shaping communities. In this experiment, salinity had no effect on the time until dispersal in the interspecific interaction experiment, but dispersal occurred at lower adult densities (regardless species identity) at the higher salinity. The lack of a salinity effect is in contrast with our expectations: lower salinity shortened time until dispersal in monospecific experiments (De Meester, et al., 2012; chapter VI), and increased interspecific interactions (De Meester, et al., 2011; chapter II). Hence, we had expected faster dispersal at the lower salinity. When competition and salinity act in concert, interactive effects between them can occur and may influence dispersal in unpredictable ways (Ims & Hjermann, 2001). Lower

salinity did not lead to stronger interactions between the species in this experiment; dispersal and competition probably influence each other more than salinity does. Our results suggest that there is a complex relationship between dispersal and biotic and abiotic factors, and a simple combined effect of these two factors on dispersal does not occur.

Some differences in the assemblage composition between the salinities were also observed, with a higher relative abundance of Pm III adults at the lower salinity, which is in agreement with higher abundances of this species compared with the other species in monospecific cultures at the lower salinity (Table 1, De Meester, et al. 2011 (chapter II)).

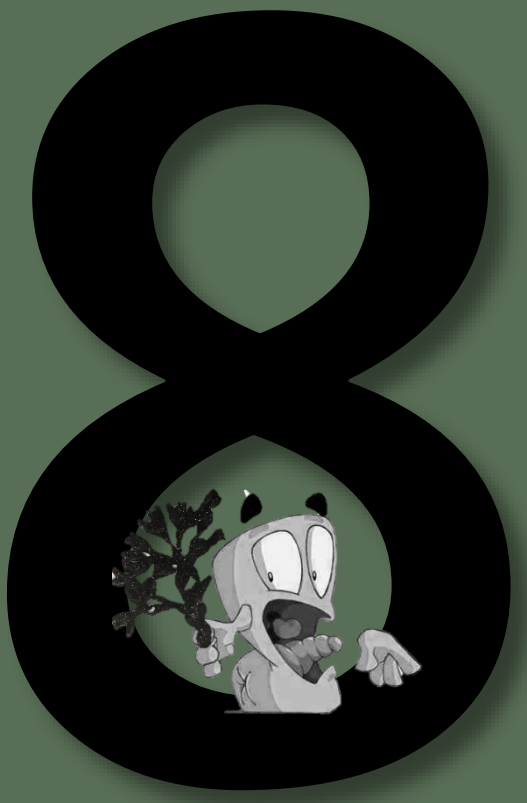
**General conclusions**

The present results demonstrate that species-specific differences in density-dependent dispersal exist in the *L. "marina"* cryptic species complex, as was also found in marine harpacticoid copepods (Service & Bell, 1987). Intraspecific competition was an important trigger for dispersal in at least two of the four cryptic nematode species (Pm III and Pm IV), while interspecific interactions were mainly important for Pm I. The effect of salinity on the co-occurrence-dispersal dynamics was less important. We also provide strong evidence that dispersal can be an important mechanism to achieve co-occurrence and perhaps coexistence. In a spatially homogeneous competitive environment (Amarasekare, 2003), we expect that (1) a colonization-competition trade-off – in which the weaker competitors, Pm II and Pm IV, are the fastest dispersers (De Meester, et al., 2011; chapter II) – is the main mechanism for coexistence. However, the current experiment shows that their dispersal abilities do not differ from those of the competitively stronger species, Pm I and Pm III. Moreover, Pm III, a strong competitor, showed a high reproductive output (De Meester, et al., 2015a; chapter III) and fast dispersal (De Meester, et al., 2012; chapter VI), so proved to be a good colonizer. Hence, a clear competition-colonization trade-off was not found in the *L. "marina"* species complex. Moreover, competition outcomes differed with salinity (De Meester, et al., 2011; chapter II), indicating that a heterogeneous competitive environment is more realistic for the *L. "marina"* species complex. In such an environment, (2) spatial storage may be the principal driver for regional coexistence. In a heterogeneous habitat, where patches vary spatially and temporally (i.e. decomposing patches of macroalgae for the *L. "marina"* complex), differential dispersal strategies are likely to evolve among closely related species (McPeek & Holt, 1992) and coexistence can arise when species disperse before the natal sites turn unfavourable as a result of (over)crowding (Snyder & Chesson, 2003). More information on abiotic preferences and niche differences between the species is needed to

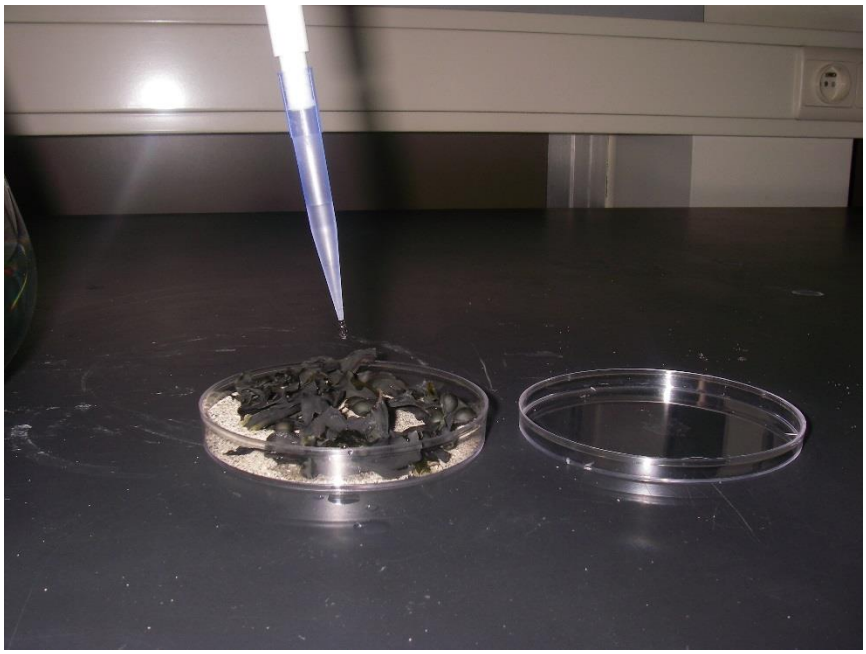
confirm the applicability of this hypothesis to the *L. "marina"* species complex. In addition, (3) neutral dynamics may be important for the coexistence of the *Litoditis* species under our laboratory conditions as no differential effect was found between interspecific and intraspecific competition for three of the four cryptic species. In this case, metacommuntiy paradigms (regional coexistence) depend on the scales of dispersal and environmental heterogeneity (Snyder & Chesson, 2003).

## Acknowledgements

Funding for this research was obtained from the Flemish Fund for Scientific Research (FWO) through project 3G040407, and from Ghent University through projects 011060002 and 01GA1911 W. S.D. is a postdoctoral fellow with the FWO.



# CHAPTER VIII



# CRYPTIC DIVERSITY AND ECOSYSTEM FUNCTIONING

---

Accepted as:

De Meester, N., Gingold R., Derycke, S., Rigaux, A. & Moens, T. Cryptic diversity and ecosystem functioning: a complex tale of differential effects on decomposition, *Oecologia.*

## Abstract

Marine ecosystems are experiencing accelerating population and species loss. Some ecosystem functions are decreasing and there is growing interest in the link between biodiversity and ecosystem functioning. The role of cryptic (morphologically identical but genetically distinct) species in this biodiversity–ecosystem functioning link is unclear and has not yet been formally tested. We tested if there is a differential effect of four cryptic species of the bacterivorous nematode *Litoditis "marina"* on the decomposition process of macroalgae. Bacterivorous nematodes can stimulate or slow down bacterial activity as well as modify the bacterial assemblage composition. Moreover, we tested if interspecific interactions among the four cryptic species influence the decomposition process. A laboratory experiment with both mono- and multispecific nematode cultures was conducted, and loss of organic matter and the activity of two key extracellular enzymes for the degradation of phytodetritus were assessed. *L. "marina"* will mainly influences qualitative aspects of the decomposition process rather than its overall rate: an effect of the nematodes on the enzymatic activities became manifest, although no clear nematode effect on bulk organic matter weight loss was found. We also demonstrated that species-specific effects on the decomposition process existed. Combining the four cryptic species resulted in high competition, with one dominant species (Pm I), but without complete exclusion of other species. Nevertheless, the effects on the decomposition process was different in these combined cultures compared with cultures where Pm I occurred alone. The interspecific interactions translated into different effects on the decomposition process. The species-specific differences indicated that each cryptic species may play an important and distinct role in ecosystem functioning. Functional differences may result in coexistence among very similar species.

## Introduction

Marine benthic ecosystems provide a range of ecosystem services, such as the regulation of climate, nutrient budgets and primary productivity (Covich, et al., 2004; Solan, et al., 2004; Costanza, et al., 2007). Marine ecosystems are experiencing accelerating population and species loss (Solan, et al., 2004; Díaz, et al., 2006; Worm, et al., 2006), which has led to an increasing interest in the relationship between ecosystem services and species richness. Ecosystem functions such as resource gains, recovery potential and ecosystem stability have already been shown to decrease with declining species and/or genetic diversity (Loreau, et al., 2001; Worm, et al., 2006). The main importance of high biodiversity is that it provides insurance against functional impairment when some species go extinct due to, e.g., environmental stress (Yachi & Loreau, 1999). This is stated by the redundancy hypothesis: the role of many species can be easily taken over by other functionally similar species (Walker, 1992b; Lawton & Brown, 1994) without substantially altering ecosystem functioning; hence, a limited number of "key" species would be sufficient to maintain ecosystem functioning (Yachi & Loreau, 1999).

The role of cryptic (i.e. morphologically identical but genetically distinct) species in the biodiversity-ecosystem functioning relationship is complicated to assess. In the past, cryptic species have often been considered as one species and their role has hitherto been neglected in biodiversity–ecosystem functioning studies (Finlay, et al., 1997; Ettema, 1998; Loreau, et al., 2001; Cardinale, et al., 2002; Danovaro, et al., 2008). Despite their morphological similarity, many cryptic species show different ecological characteristics (Ortells, et al., 2003; Gerhardt, 2005; De Meester, et al., 2015a (chapter III); Fišer, et al., 2015) and, as a consequence, may display different ecological functions, at least at a local scale (Fišer, et al., 2015). Moreover, the sympatric occurrence of many cryptic species (e.g. Pinto, et al., 1986; Trewick, 1998; Mayer & Von Helversen, 2001; Ortells, et al., 2003; Zhang, et al., 2004; Derycke, et al., 2006; Amato, et al., 2007; Wellborn & Cothran, 2007) indicates that differences among them may be more pronounced than presumed based on their morphological similarity. Coexistence requires at least some ecological differences among species, which may lead to differences in ecosystem functioning (Loreau, 2004) (but see Hubbell, 2001 for the neutral theory of coexistence). Nevertheless, no experimental studies have hitherto been conducted to test if differences in ecosystem functioning exist among cryptic species.

Cryptic species have been observed in several nematode genera (Sudhaus & Kiontke, 2007; Derycke, et al., 2013), but it remains unclear whether there are functional differences among them. Free-living marine nematodes are commonly assigned to a limited number of feeding guilds (Moens & Vincx, 1997; Moens, et al., 2004). Species pertaining to one guild, as do cryptic species, are often assumed to be functionally redundant. Nevertheless, differences in (micro-)habitat preference, in resource use, and in impacts on microbial assemblages among species within a guild, a family, or even a genus cast doubts on the applicability of the concept of redundancy to the often species-rich nematode assemblages (Ettema, 1998; Wolters, 2001; De Mesel, et al., 2004; Vafeiadou, et al., 2014).

Benthic ecosystems depend on the supply of organic material, most of which enters the sediments as polymeric organic compounds (Meyer-Reil, 1987). Through the production of extracellular enzymes bacteria convert polymeric compounds into smaller, assimilable molecules (Chróst, 1991). The enzymes Leucine-aminopeptidase and β-glucosidase play key roles in this process (Danovaro, et al., 2002). Nematodes can stimulate or slow down bacterial activity (Findlay & Tenore, 1982; Alkemade, et al., 1992; 1993; Mamilov, et al., 2000; De Mesel, et al., 2003; Urban-Malinga, et al., 2008) as well as affect bacterial assemblage composition (De Mesel, et al., 2004) and may thus play a key role in decomposition processes and nutrient regulation (Abrams & Mitchell, 1980; Yeates & Coleman, 1982; Freckman, 1988; Neher, 2001). Different nematode species belonging to the same functional group and even to the same family can have distinct influences on decomposition processes and can thus not be considered functionally redundant (De Mesel, et al., 2006).

The cryptic species complex of the bacterivorous nematode *Litoditis "marina"* (Sudhaus, 2011) (formerly known as *Rhabditis marina* or *Pellioditis marina*) consists of at least ten cryptic species (Derycke, et al., 2008a). Four of these cryptic *L. "marina"* species (Pm I, Pm II, Pm III and Pm IV) occur frequently in the littoral zone of the south-western coast and estuaries of The Netherlands (Derycke, et al., 2006; 2008b). Sympatric occurrence of two or more of these species on decomposing algae is in the rule rather than exception (Derycke *et al*., 2008b; Derycke *et al*., 2005). The cryptic species lack distinctive morphological differences, but show molecular divergences at both nuclear and mitochondrial loci (COI, ITS, D2D3); cross breeding between the two most closely related species (Pm I and Pm IV) has not been detected (Derycke, et al., 2008a; Fonseca, et al., 2008). Competitive interactions among the species are common, and abiotic conditions can change the outcome of these

interactions (De Meester, et al., 2011 (chapter II); 2015c). Moreover, there are other ecological differences among the species, such as differences in dispersal rates and different population responses to temperature and salinity conditions (De Meester, et al., 2011 (chapter II); 2012 (chapter VI); 2015b (chapter VII)). Whether such differences have functional implications remains to be established.

In the present study, we investigated the effect of cryptic species on the decomposition rate by assessing loss of organic matter and the activity of two key extracellular enzymes (β-glucosidase and Leucine-aminopeptidase) for the degradation of phytodetritus. In a microcosm experiment, we tested whether functional differences among cryptic species exist. The coexistence of many cryptic species led us to hypothesize that cryptic species would have different influences on microbial enzymatic activity and on organic-matter decomposition rate. Given that Pm III is genetically (Derycke, et al., 2008a; Grosemans, et al., 2016) and ecologically (De Meester, et al., 2012 (chapter VI); 2015a (chapter III)) more distinct from the other three species, we expected that functional differences would be most pronounced between Pm III and the other cryptic species. We also tested whether a combination of all four cryptic species of *L. "marina"* would differentially affect the microbial activity and the decomposition rate of organic matter compared to single-species treatments. We expected high competition between the cryptic species (De Meester, et al., 2011; chapter II) and that these interspecific interactions may result in species switching their type of food to avoid competition (Tilman 1976; Al-Naimi et al. 2005; Postma-Blaauw et al. 2005), influencing bacterial composition and as a consequence also the decomposition process.

## Material & Methods

### Nematode cultures

For the experiments, nematodes of *Litoditis "marina"* (Fig. 1) were harvested from monospecific stock cultures in exponential growth phase. Monospecific cultures of four different cryptic species (Pm I, Pm II, Pm III and Pm IV) were raised from single gravid females obtained from the field (for Pm I, Pm II and Pm III: Paulina marsh, Westerschelde, The Netherlands; for Pm IV: Lake Grevelingen, The Netherlands) and maintained on sloppy (0.8%) nutrient:bacto agar media (temperature: 20°C; salinity: 25) with unidentified bacteria from their habitat as food (Moens & Vincx, 1998).

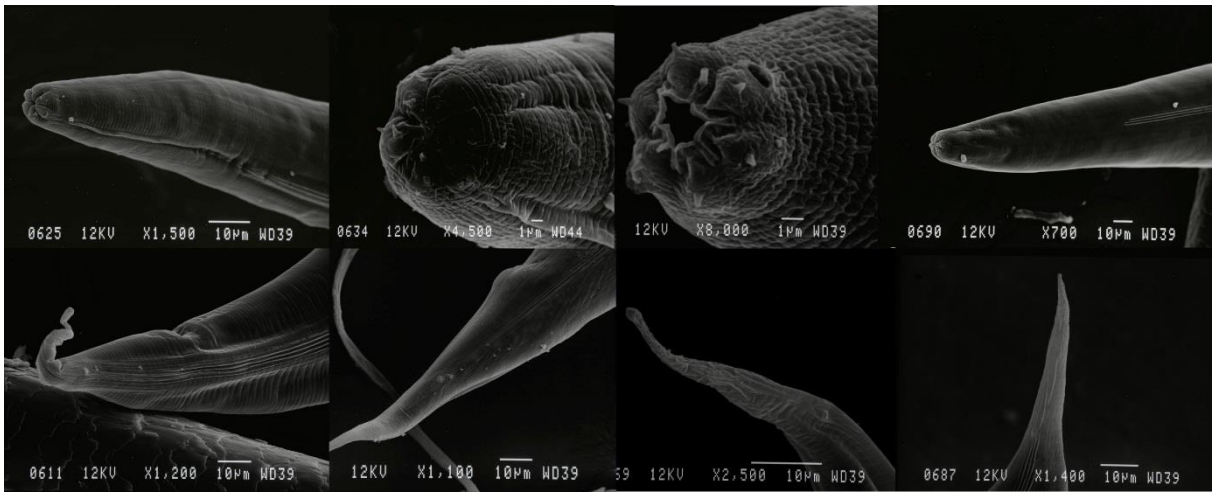



Figure 1: Scanning-electron microscopy pictures of four cryptic species of *Litoditis "marina"*. From left to right: females of Pm I, Pm II, Pm III and Pm IV. Upper row: the nematode head; lower row: a part of the tail, showing the shape of the tail tip (pictures provided by Derycke S.).

### Experimental setup

To test the species-specific effect on decomposition, we compared four monospecific treatments (Pm I, Pm II, Pm III and Pm IV referred to with treatment codes M1, M2, M3 and M4, respectively) using inoculations of 20 nematodes (12 females and 8 males) in closed microcosms (petri dishes with an inner diameter of 8.5 cm). To test the effect of cryptic biodiversity and interspecific interactions on decomposition, a combination treatment with all four cryptic species (coded All4) was included following a substitutive design (with 3 females and 2 males per species, hence the same total number of nematodes as in the M treatments). A control (C) without nematodes was also included to assess to what extent the presence of nematodes affected decomposition rate. The microcosms consisted of sediment

with macro-algal thalli (*Fucus sp.*) that had been collected at the Paulina mudflat, Westerschelde Estuary (The Netherlands). The sediment was defaunated by first washing it over sieves of 1 mm and 38 µm and then drying it at 110°C for three days. Observation under a stereomicroscope, after five decantation steps, was used to confirm that no living nematodes were present in the defaunated sediment. Algae were submerged in artificial seawater (ASW, salinity of 20) for three days to allow initial leaching of nutrients (preliminary tests had shown excessive microbial growth on algal thalli that had not received this treatment), then carefully washed with tap water, and finally dried at 60°C for two days. Each microcosm consisted of the same amount of sediment (20 g dry weight) and algae (2 g dry weight). Four replicate microcosms were made for every treatment and for each of five time points (see below), resulting in a total of 120 microcosms (20 microcosms per treatment plus 20 controls).

Microcosms were water saturated with 10 mL of ASW, and 250 µL of a bacterial inoculum was added. This bacterial inoculum consisted of a bacterial mixture from the different nematode stock cultures and from the field: for the latter, freshly collected algae were soaked in ASW for five days, and 50 mL of the resulting suspension was mixed with a small piece of agar from each nematode stock culture. The mixture was well shaken and filtered over a 0.8 µm Millipore filter to remove fungi, flagellates, ciliates, nematodes. ASW was added to reach a final volume of 60 mL. The experiment was run over 25 days, which is the approximate time of at least five *L. "marina"* generations. Sampling over a sufficiently long period ensures that the nematode population development can be linked to the decomposition process.

**Sampling procedure**

Every fifth day, one set of four experimental replicates and one control were processed for each treatment. First, algae were picked from the microcosms and were put in 25 mL of ASW in a Falcon tube and stirred gently. Subsequently, we also carefully rinsed the algae with tap water over a 32 µm sieve to collect the nematodes from the algae. Of the resulting suspension (35 mL, from the stirring and the rinsing step), two 1-mL samples were immediately stored at –80°C for later enzymatic assays. The algae were dried at 60°C for two days, weighed, and the percentage of weight loss of organic matter was calculated. The water with the collected nematodes was sieved over a 32 µm sieve, and the nematodes were stored in DESS (Yoder, et al., 2006)

About 1 g of sediment was used for enzymatic analysis. The rest of the sediment was stored in DESS for later nematode counts and identifications. We conducted separate analyses of nematodes from algae and sediments, because nematodes may disperse to either substrate when they are subject to intra- or interspecific competition (De Meester, et al., 2015b; chapter VII).

**Nematode counts and identification**

Sediment samples in DESS were sieved over a 32 µm sieve and collected in tap water. Five decantation steps were performed to collect the nematodes. Three subsamples of 1 mL (out of 35 mL) were taken and juvenile and adult nematodes were counted under a stereomicroscope. The distinction between adults and juveniles is important because (1) competitively weaker species may largely disappear from the adult population but remain present as juveniles or dauer stages, and (2) the feeding behaviour between juveniles and adults may differ; thus leading to differential effects on decomposition. Total nematode abundances were calculated by multiplying the average of the three subsamples with the total volume of the sample. Because 1 g of sediment and 2 mL of algae were removed for enzymatic analysis before counting the nematodes, the total number of nematodes was recalculated to the original weight of the sediment (20 g) or volume of water (35 mL).

To obtain a relative quantification of each cryptic species in the All4-treatment, we performed a quantitative real-time PCR analysis (qPCR) (De Meester, et al., 2015b; chapter VII). DNA was prepared by adding hexadecyltrimethylammonium-bromide (CTAB) to the volume needed for ca. 100 nematodes. The concentration of DNA was measured with the Nanodrop 2000 (Isogen Life Science) and concentrations above 10ng $\mu L^{-1}$ were diluted. Identifications of the cryptic species were performed using the Lightcycler 480 System and the SensiMix SYBR No-ROX Kit (Bioline). The qPCR mixture was prepared for a 10 µL reaction volume on 384-well plates using 5 µL SensiMix SYBR No-ROX One-Step (2x) solution, 1 µL PCR-grade water, 1 µL of DNA template and 3 µL of each primer. Species-specific primers were developed in the ribosomal internal transcribed spacer region (ITS) and were used with final concentrations of 1 µM for Pm I and Pm III, 500 nM for Pm II and 200 nM for Pm IV (protocol as described in Derycke, et al., 2012). The thermal cycling protocol consisted of an initial denaturation for 10 min at 95°C followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 20 s at 60°C and extension for 20 s at 72°C. Two technical replicates per sample were conducted. For the assemblage composition differences

in Ct values were calculated using the adjusted ΔΔCT method (Livak & Schmittgen, 2001; Derycke, et al., 2012).

**Assessment of decomposition rate and enzymatic activity**

Nematode-free residuals of the sediment were collected per sample, dried at 60°C for two days and weighted, similar as for the algae (see above). Microbial exo-enzymatic activities (β-glucosidase and Leucine-aminopeptidase) in sediment and on algae were measured with fluorescence following the protocol of Meyer-Reil (1987) and Danovaro, et al. (2002) . In short, 1 g wet weight of sediment or 1 mL of the algae suspension was transferred to a 15-mL falcon tube to which 5 mL of ASW were added. Substrate stock solutions with increasing fluorogenic substrate concentrations were prepared in cellosolve by adding 150 µL of 4-methylumbelliferyl-β-D-glucoside and L-leucine-4-methylcoumarinil-7-amide-hydrochloride (final concentration of 100 µM). These substrate stock solutions (10 µL) were added to the samples and were gently shaken. We used 100 µL of these samples for measuring the exo-enzymatic activities. Sample fluorescence was measured once for each sample for 0.1 s on a Victor$^3$ Multilabel Reader (PerkinElmer) with the Umbelliferone program (excitation 355 nm/ emission 460 nm) immediately after substrate inoculation to assess the "background value". Afterwards, samples were incubated for one hour in the dark, centrifuged for 10 minutes at 2500 rpm and fluorescence was measured again. The background value was subtracted from fluorescence after 1h of incubation and enzymatic activity was standardized per unit dry weight of sediment or algae.

**Statistical analyses**

All analyses were conducted in R (R Development Core Team 2008). Different statistical models were built for the dependent variables nematode density and enzymatic activity, based on the data distribution and on the correlation between algae and sediment by use of package nlme. For weight loss and species composition, respectively ANOVA and PERMANOVAs (by use of package vegan) were conducted. An overview of all the final models can be found in Table 1.

Table 1: Overview of all statistical models/tests

| Test variables | Statistical model/test |
|---|---|
| Nematode density | Weighted linear mixed model |
| Weight loss | ANOVA |
| Enzyme activity | General linear model |
| Correlation between nematode density and weight loss/enzyme activity | Spearman's rank correlations |
| Species composition | PERMANOVA |

### *Species effect on the decomposition process*

Whether species have differential effects on the decomposition process and whether a combination of species could alter this effect was tested by assessing weight loss of organic matter and enzymatic activity in mono- and multispecies treatments.

*A. Weight loss of organic matter*

The effect of time, treatment and their interaction on the percentage weight loss was tested with a two-way ANOVA. To meet the assumptions of normality and homoscedasticity, the data were log transformed. Pairwise comparisons were conducted with a post-hoc Tukey Honest Significant Differences (HSD) test on the significant terms and interactions.

*B. Enzymatic activity*

The effect of substrate (algae or sediment), time (day 5, 10, 15, 20 and 25) and treatment (Pm I, Pm II, Pm III, Pm IV and All4) on enzymatic activities was assessed separately for β-glucosidase and Leucine-aminopeptidase by a general linear model (Table 2). To achieve randomly scattered residuals against the fitted values, the data were square root transformed. Different models were created: a general linear model, a random intercept model, the random intercept and slope model, and the random effects model (Zuur, et al., 2009). Akaike Information Criteria (AIC) (Akaike, 1981) were used to select the most appropriate model. No significant correlation of enzymatic activities on algae and in sediment was found; therefore the General Linear Model turned out to be the best model (Table 2). In a step-wise backward procedure, non-significant factors were removed from the model. Pairwise comparisons on the significant factors were done by dummy coding in the models (Suits, 1957), followed by a Bonferroni correction.

***Effect of nematode densities on the decomposition process***

Numbers of nematodes may differ between the different treatments and any observed effect on the decomposition process may therefore be the result of different nematode densities rather than of species identity or species composition. Therefore, we first investigated the numbers of nematodes per treatment and over time, and analysed the correlations between these numbers and the decomposition process.

*A. Nematode densities*

We used weighted linear mixed models to test for the effects of substrate (algae or sediment), time (day 5, 10, 15, 20 and 25) and treatment (Pm I, Pm II, Pm III, Pm IV and All4) on the number of adults and the number of juveniles, because the assumption for homogeneity of the residuals was not met. An exploration of the residuals showed that their spread differed per level of the different factors, so different models with different variance structures (for all main factors and all interactions) were created. Moreover, the number of nematodes (both juveniles and adults) on algae was significantly correlated with the number of nematodes in the sediments, so sample ID was included as a random factor in the models and different models with this random factor were created (random intercept model, the random intercept and slope model, and the random effects model (Zuur, et al., 2009)). AIC were used to select the most appropriate model, which, in this case, turned out to be a weighted linear mixed model with random intercept and the three-way interaction defined as variance structure (Table 2). Pairwise comparisons were done by dummy coding followed by a Bonferroni correction.

*B. Correlations between enzymatic activity/weight loss and number of nematodes*

To test whether the number of nematodes was correlated with enzymatic activity and weight loss of organic matter, Spearman's rank correlations were calculated as normality of the data was not achieved, even after transformations. Correlations were tested for each treatment separately (Pm I, Pm II, Pm III, Pm IV and All4). Moreover, we added nematode density (after log-transformation) as covariate in the General Linear Model for enzymatic activity, to test if nematode density affect enzymatic activity.

Table 2: Overview of the final statistical models for nematode density and enzyme activity.

| Model made for | Dependent variable | Trans-formation used | Type of model | Independent factors included in the model | Random factor | Weighted factor |
|---|---|---|---|---|---|---|
| Density | Number of adults | None | Weighted Linear mixed model | substrate, time, treatment and all interactions | sample | Substrate:treatment: time |
| Density | Number of juveniles | None | Weighted Linear mixed model | substrate, time, treatment and all interactions | sample | Substrate:treatment: time |
| Enzyme | Glucosidase activity | Square root | General linear model | substrate, time, treatment and all interactions | none | none |
| Enzyme | Aminopeptidase activity | Square root | General linear model | substrate, time, treatment, substrate:treatment, treatment:time | none | none |

***Effect of interspecific interactions on species composition (All4 treatment)***

To test whether interspecific interactions had an effect on the species composition of the All4 treatment, a fictitious assemblage (F) per time moment and substrate was constructed by using the relative abundances of each species in the monospecific treatments (M1 + M2 + M3 + M4). As such, these fictitious assemblages reflect species composition without interspecific interactions. These were compared with the "real" assemblages of the All4 treatment (R), where interspecific interactions were present, by the use of a PERMANOVA, conducted in R using the Adonis function (Vegan package) (Oksanen, et al., 2007). The independent fixed factors were substrate, time, and interspecific interaction treatment (no interaction (fictitious) vs. real assemblage (All4 treatment)). The analyses were performed on the adult and juvenile assemblage compositions separately. The interdependency of the assemblages on algae and sediment of one sample was taken into account in the test (by adding the sample ID as a random factor). A Bray-Curtis dissimilarity index was used and 999 permutations were conducted. The assumption of homogeneity of group dispersions was investigated using the betadisper command. In this way we were able to test whether significant factors only influenced assemblage composition or also the variation among the different replicates. Significant terms and interactions were investigated using posterior pair-wise comparisons with a Bonferroni correction. A SIMPER analysis in R was used to identify which species primarily accounted for the observed differences. Due to the

differences in start densities (80 nematodes for the fictitious populations and 20 for the real populations), no comparisons between total numbers of each nematode species in the real and fictitious assemblages were conducted.

## Results

### Species effect on the decomposition process

*A. Weight loss of organic matter*

There were significant differences in weight loss of organic matter among the different sampling times ($F_{20,90}$ = 12.47, p=4.10e$^{-8}$), with higher weight loss from day 10 onwards. There was, however, no difference in weight loss of organic matter among the different species (treatments), or between the treatments and the control group without nematodes (ANOVA, all p> 0.05 (Appendix 1)).

*B. Enzymatic activity*

β-glucosidase activity was significantly different between treatments and substrates, and all interaction terms were significant (Table 3, Fig. 2A and B). β-glucosidase activity differed between algae and sediment only at some time moments (Fig. 2) and was in general higher on the algae compared to the sediments. In the sediment, there was no species-specific effect on β-glucosidase activity at any time moment (Fig. 2A). By contrast, species-specific differences in β-glucosidase activity were found on the algae: after 10 days, all monospecific treatments, except Pm IV, showed lower enzymatic activity compared to the control, and the treatment with Pm I exhibited the lowest activity of all (all p<0.006). After 20 days, the Pm IV treatment produced the highest enzymatic activity, and Pm I also showed higher enzymatic activity compared to the control (all p<0.035). Yet, after 25 days, the Pm I treatment again yielded the lowest enzymatic activity compared to all other treatments (all p<0.001) (Fig. 2B).

Leucine-aminopeptidase activity was influenced by substrate, time, treatment, the interaction of substrate with treatment, and the interaction between time and treatment (Table 3). Leucine-aminopeptidase activities differed among the treatments (regardless substrate) only after 25 days: the Pm I treatment had lower enzymatic activities compared with the treatments Pm III, Pm IV and All4; the Pm II treatment showed lower enzymatic activity compared to the monospecific treatments of Pm III and Pm IV. Moreover, the Pm III treatment had a significantly higher enzymatic activity than the control. Within the sediment,

no differences were found among the different treatments. Leucine-aminopeptidase activity was higher on the algae compared to the sediment for the control, Pm III, and All4 (Fig. 2C and D). Within the treatments, no differences were found among the time moments except for the treatments of Pm III (day 25 had a higher activity than all the rest), Pm IV (higher activity at day 25 compared to day 10 and 20) and All4 (higher activity at day 25 compared to day 5) (Fig. 2C and D).

Table 3: Results of the general linear model on enzymatic activity of β-glucosidase and Leucine-aminopeptidase

| Factor | | β-glucosidase | | Leucine-aminopeptidase | |
|---|---|---|---|---|---|
| | Df | F | P | F | P |
| Substrate[1] | 1 | 867.28 | **<0.0001** | 52.67 | **<0.0001** |
| Time[2] | 4 | 1.26 | 0.29 | 2.63 | **0.04** |
| Treatment[3] | 4 | 3.98 | **0.0026** | 2.93 | **0.01** |
| Substrate*Time | 4 | 3.08 | **0.02** | not included in final model | |
| Substrate* Treatment | 4 | 3.49 | **0.0063** | 2.66 | **0.02** |
| Time*Treatment | 16 | 3.17 | **0.0001** | 2.96 | **0.0001** |
| Substrate*Time*Treatment | 16 | 3.45 | **<0.0001** | not included in final model | |

[1]algae, sediment ;[2]day 5, 10, 15, 20 and 25; [3] Pm I, Pm II, Pm III, Pm IV and All4

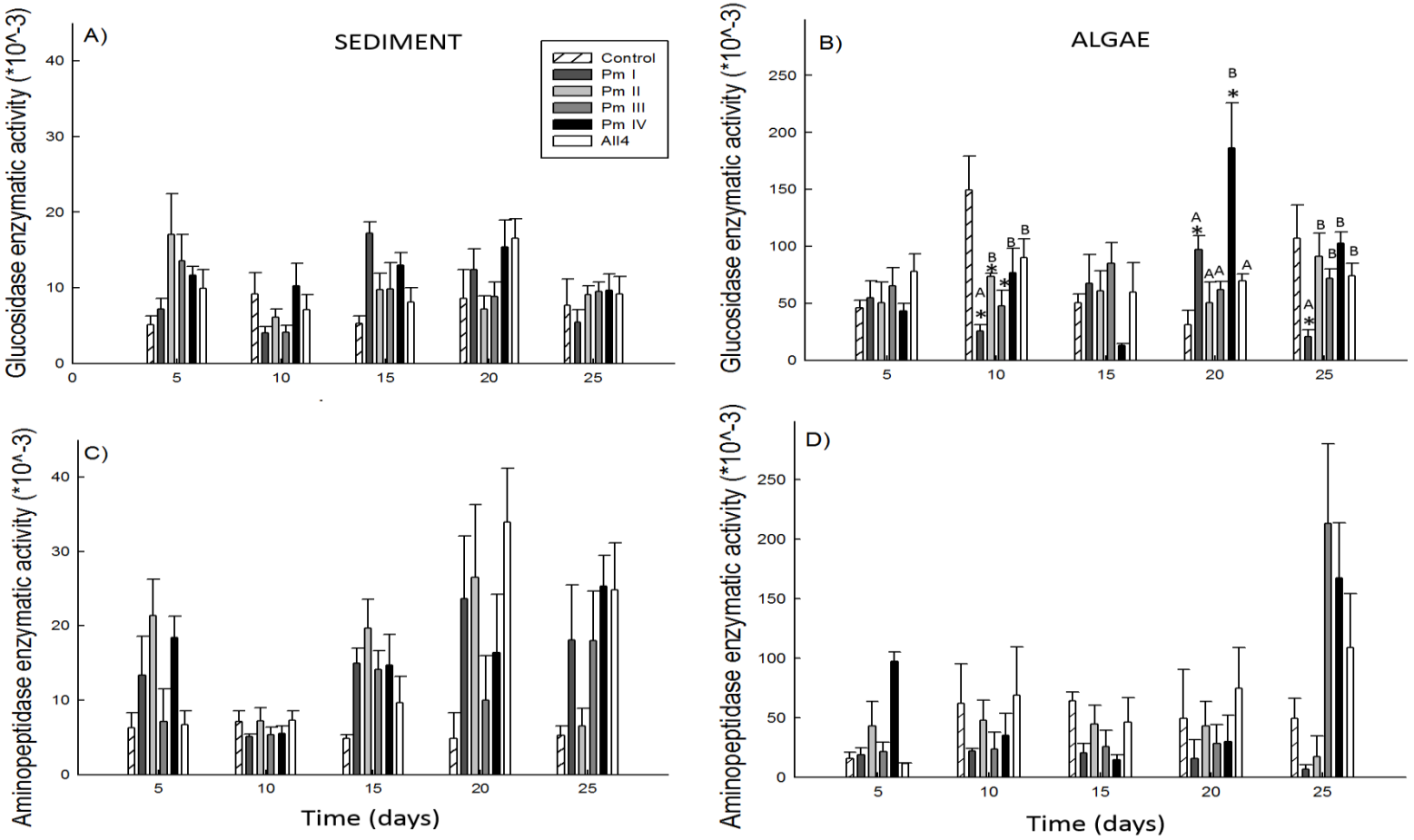



Figure 2: Enzymatic activities (mean + SE of 4 replicates) over the different treatments for β-glucosidase on a) the sediment and b) the algae and for Leucine-aminopeptidase on c) the sediment and d) the algae. Different letters show significant pair-wise differences of the three-way interaction between treatments, * show significant pair-wise differences with the control. For leucine-aminopeptidase letters are absent, because the tree-way interaction was not significant. Attention: the y-axes have different scales for sediment and algae. (n=100)

## Effect of nematode densities on the decomposition process

### *A. Nematode densities*

During the course of the experiment, all species had very similar abundances and none of the species performed remarkably better than the others (Appendix 2, fig. S2) . Nevertheless, some species-specific significant differences, depending on substrate and time, existed: Pm I showed highest adult abundances on the algae at day 5 and in the sediment at day 25 compared with some other treatments. Pm II exhibited higher adult and juvenile abundances on the algae at day 20, but lower juvenile abundances at day 25 in the sediment. Pm III showed higher adult abundances at day 25 on the algae and higher juvenile abundances at that day in the sediment. At day 25, All4 showed higher abundances of juveniles on the algae compared with some other treatments. More details on nematode densities and their differences between treatments can be found in Appendix 2.

### *B. Correlations between enzymatic activity/weight loss and number of nematodes*

No significant correlations were found between weight loss and number of nematodes. Significant correlations were found between enzymatic activity and number of nematodes: in the sediment, the number of juvenile Pm I was significantly correlated with both β-glucosidase ($r=0.59$; $p=0.006$) and Leucine-aminopeptidase ($r=0.47$; $p=0.035$). On the algae, the numbers of juvenile and adult Pm III were negatively correlated with β-glucosidase activity ($r=-0.53$; $p=0.015$ and $r=-0.48$; $p=0.03$, respectively). In the General Linear Model on enzymatic activity, no significant interaction with nematode density was found. After the step-wise backward procedure, nematode density was completely deleted from the model.

**Effect of interspecific interactions on species composition (All4 treatment)**

The assemblage composition of adults was influenced by all factors except the three-way interaction (Table 4). Time did not only influence assemblage composition but also the variation among the different replicates. At every time moment, adult assemblages differed between the real and the fictitious populations, with Pm I being the most dominant species in all real assemblages (explained at each time moment more than 57% of the variation (SIMPER)). Pm I was always the most abundant species, but never completely excluded the other species, which had variable relative abundances over time. By contrast, a more even distribution of all species was found in the fictitious populations (Fig. 3A and B). These differences were seen both on the algae and in the sediment. Some differences existed also between substrates: Pm I was more abundant on the algae compared to the sediment, while at day 5, Pm IV was less abundant on the algae compared to the sediment; the same was true for Pm II at day 10. In both the fictitious and the real assemblages, there were differences in species composition between the algae and in sediment. Pm I was less abundant on the algae in the real assemblages, whereas the opposite was true in the fictitious assemblages.

The assemblage composition of juveniles gave very similar results, except for the interaction of interspecific interaction and substrate, which was not significant for the juveniles (Table 4). Assemblage compositions differed between fictitious and real populations at all time moments in the same way as for the adults, except at day 5, where the assemblage composition was very similar both in presence and absence of interspecific interactions (Fig. 3C and D). Moreover, assemblage composition did mostly differ between the start and the rest of the experiment, and was very similar from day 15 onwards for fictitious assemblages and from day 10 onwards for the real assemblages (Fig. 3C and D).

Table 4: Results of the PERMANOVA on adult species composition (All4 treatment)

| Factor | | Adults | | Juveniles | |
|---|---|---|---|---|---|
| | Df | pseudo-F | p | pseudo-F | p |
| Substrate[1] | 1 | 2.72 | **0.002** | 4.26 | **0.001** |
| Time[2] | 4 | 19.97 | **0.001** | 20.75 | **0.001** |
| Intersp. int.[3] | 4 | 16.13 | **0.001** | 16.50 | **0.001** |
| Substrate*Time | 4 | 2.61 | **0.001** | 2.93 | **0.001** |
| Substrate*Intersp. int. | 4 | 2.22 | **0.003** | 0.55 | 0.239 |
| Time*Intersp. int. | 16 | 3.75 | **0.001** | 3.82 | **0.001** |
| Substrate*Time*Intersp. int. | 16 | 0.59 | 0.575 | 0.57 | 0.295 |

[1]algae, sediment ;[2]day 5, 10, 15, 20 and 25; [3] interspecific interaction: real and fictitious

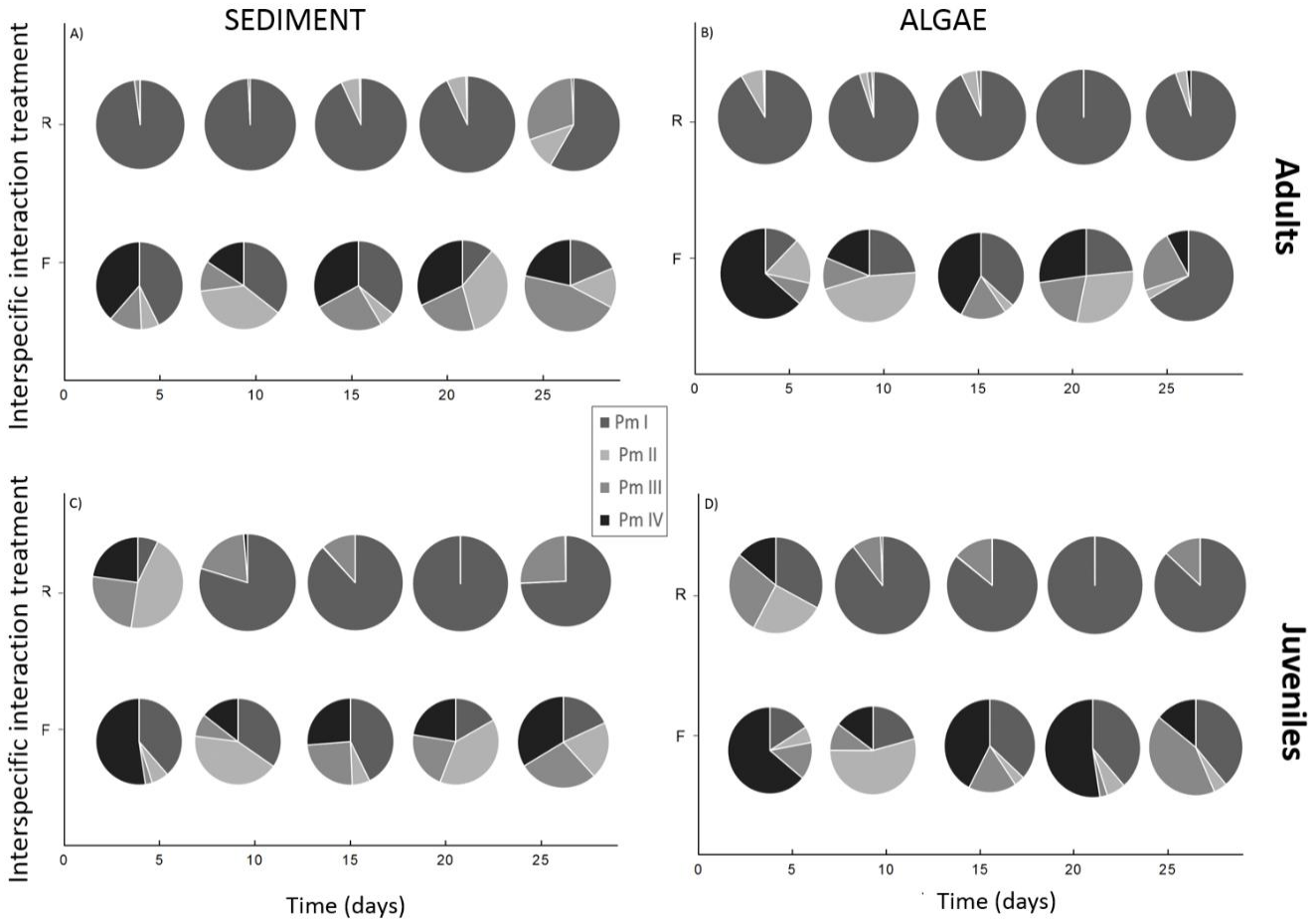



Figure 3: Average number of nematodes (mean ± SE) over time in the real (R) vs. fictitious (F) assemblages on the different substrates (sediment and algae) for adults (a and b) and juveniles (c and d). Pie charts show the assemblage composition of the cryptic species complex of *L. "marina"* at day 5, 10, 15, 20 and 25. (n=80)

## Discussion

Our study shows that the members of the bacterivorous nematode *Litoditis "marina"* complex influence enzymatic activities in a species-specific manner. This did not translate into an effect on weight loss, indicating only a possible qualitative effect on decomposition. In addition, interspecific interactions influenced species composition, with Pm I being the most dominant species. These interspecific interactions also influenced the decomposition process.

### *Litoditis "marina"* nematodes have a qualitative effect on decomposition

In our experiment, an effect of the nematodes on the enzymatic activities manifested after ten days, but no clear nematode effect on organic matter weight loss was found, which suggests that *L. "marina"* mainly influences qualitative aspects of the decomposition process rather than its overall rate or that the effect on weight loss will only manifest later on. Every cryptic species, except Pm IV, negatively influenced the enzymatic activity on at least one time moment compared to the control (see below for more details on species-specific effects). Although this was statistically significant for β-glucosidase but not for Leucine-aminopeptidase, very similar trends were visible for both enzymes, except in the Pm III treatment. This negative effect on enzymatic activity strongly suggests a top-down effect of the nematodes on the bacteria, probably through grazing leading to lower bacterial densities, which translate into lower enzymatic activities. Previous research has already shown such a relationship between bacteria and marine nematodes (De Mesel, et al., 2003). Unfortunately, our experiment did not include counts of bacterial densities. While in the beginning an inhibitory effect on enzymatic activity was present, this effect disappeared later in the experiment. This may be caused by an increase of mucus trails from the nematodes (Riemann & Schrage, 1978; Moens, et al., 2005; De Mesel, et al., 2006) or an increase in ammonia excretion by the nematodes (Ferris, et al., 1998) during the course of the experiment, which may have led to an increase in bacterial density. Another possibility is that nematodes initially compete for the most nutritious bacteria or for bacteria without anti-grazing strategies (Matz and Kjelleberg 2005). Once these bacteria are depleted, less preferred bacteria may be more prominent and competition for these bacteria will be lower. As a result, the top-down effect on the bacteria will reduce. *L. "marina"* may thus influence the decomposition process, but this effect may change over time.

**Cryptic *Litoditis "marina"* species show species-specific effects on decomposition**

Species identity rather than nematode densities influenced the enzymatic activity in our experiment. If complete functional redundancy among the species existed, we would have expected the same effect on decomposition of each single species. This was clearly not true, because enzymatic activities differed among the different treatments, while at the same time nematode densities of the different cryptic species were very similar. The presence of Pm II and Pm III nematodes showed a grazing effect (i.e., lower enzymatic activity) at the start, which disappeared later. For Pm I, Pm III and Pm IV a higher enzymatic activity of one of the enzymes compared to the control was found at a later time moment. In the presence of Pm IV, enzymatic activities were never lower than the control, indicating that Pm IV may stimulate bacterial growth, rather than overgraze the bacteria. These results suggest that Pm IV may be more important in accelerating the decomposition process compared with the other species while Pm II may not have any stimulatory effect at all. Nematode species belonging to the same functional group can show differences in their influence on decomposition as a result of selective grazing (De Mesel, et al., 2003; Postma-Blaauw, et al., 2005). Recent analysis of the microbiomes of Pm I, Pm II and Pm III has shown that the cryptic species within the *L. "marina"* species complex can be dietary specialists (Derycke, et al., 2016; chapter V). The distinct species-specific differences in our study suggest that functional redundancy among the four cryptic species may be limited. As such, our results support the contention that species diversity (*sensu stricto)* affects ecosystem functioning. This agrees with the rivets hypothesis (Ehrlich & Ehrlich, 1981), which states that all species are functionally important in an ecosystem.

**Strong interspecific interactions influence the decomposition process**

Combining species resulted in strong competition between them, with Pm I being the competitively strongest species (in agreement with De Meester, et al., 2011; chapter II). Nevertheless, Pm I never completely outcompeted the other species, and after 25 days the other species again became more abundant on the algae. This contrasts with the results of a previous study (De Meester, et al. 2011; chapter II), where Pm II and Pm IV were completely excluded. More spatial habitat heterogeneity (due to the algae and sediment in this experiment compared with a homogeneous agar layer in the previous experiment) (Amarasekare & Nisbet, 2001) and/or the higher food diversity (in this experiment a mixture of different bacterial strains was used compared with *Escherichia coli* as the main food source in the previous experiment) may have decreased competitive interactions among

species in the present experiment. Pm I adults started to decrease at day 25 which was likely caused by intraspecific competition, as numbers of Pm I also decreased in the monospecific cultures when 150 000 adults per microcosm were reached. The decline of Pm I was accompanied by growing populations of the other species. Alternatively, or in addition to interspecific interactions, nematode abundances may also have been the result of the changes in bacterial communities and growth rate of bacterial strains over time (Freckman, 1988) caused by changes in the quality of the detritus. Such changes can lead to changes in the nematode composition as has also been observed for terrestrial nematodes (Wang, et al., 2004), for instance as a result of differential food preferences (see further).

Although the All4 treatment was strongly dominated by Pm I, this did not lead to the same enzymatic activities as seen in the monospecific Pm I cultures. This demonstrates that interspecific interactions and/or the presence of other cryptic species, even at low relative abundances, have an influence on the enzymatic activity. Moreover, no higher total abundances were found in the combined treatment compared to the Pm I treatment, indicating that nematode abundance *per se* is unlikely to have caused the differences in enzymatic activity. Under conditions of interspecific interactions, species may be forced to change their type of food (Tilman, 1976; Al-Naimi, et al., 2005; Postma-Blaauw, et al., 2005). In the present study, switching food (i.e. bacteria) sources may have influenced bacterial composition and, as a consequence, the decomposition process.

**Coexistence between cryptic species may be achieved by functional differences**

Despite the high competition among the cryptic species, they were able to coexist in this experiment. Coexistence in the field is also common for at least three of the four cryptic species (Derycke, et al., 2006; 2008b). Species-specific effects on decomposition may indicate some kind of niche differentiation and may help to explain their coexistence. Functional redundancy is incompatible with stable coexistence, especially at larger temporal and spatial scales. Instead, stable coexistence requires ecological differences between species (Cothran, et al., 2015), which can then lead to functional differences (Loreau, 2004). There are distinct ecological differences in competitive abilities (De Meester, et al., 2011; chapter II), dispersal capacities (De Meester, et al., 2012 (chapter VI); 2015a (chapter III)) and life-history traits (De Meester, et al., 2015b; chapter VII) among the cryptic *L. "marina"* species. Pm III is in general the most "unique" in these ecological aspects compared to the other three cryptic species, which agrees well with its greater genetic distance to the other species (Derycke, et al., 2008a; Grosemans, et al., 2016). Moreover Pm III differed from Pm

I in resource use (Derycke, et al., 2016; chapter V). It is therefore surprising that Pm III did not differ more from Pm I and Pm IV in its effect on enzymatic activity in the present experiment. The two genetically most closely related species (Pm I and Pm IV) do not show big differences in ecological characteristics (De Meester, et al., 2012 (chapter VI); 2015b (chapter VII); 2015c (chapter IV)); however, in the present study, Pm IV always showed a more positive effect on the β-glucosidase activity compared to the other species. This indicates that Pm IV is functionally more different from Pm I than anticipated and that genetic distance may be a poor predictor of functional differences.

**Conclusions**

Despite the increasing interest in unravelling the importance of cryptic species for ecosystem functioning (Pfenninger & Schwenk, 2007), previous studies did not explicitly test for functional differences among cryptic species. Our results show that cryptic species induce distinct species-specific differences in enzymatic activity, which was used here as a proxy for the decomposition process. This indicates that functional differences exist among the species and each cryptic species may play an important role in the ecosystem. These functional differences show that cryptic species differ more than first thought and differences between the species may help to explain coexistence.

## Acknowledgements

Funding for this research was obtained from the Flemish Fund for Scientific Research (FWO) through project G038715N, and from Ghent University through projects 011060002 and 01GA1911W. Annick Van Kenhove is acknowledged for help with the set-up and sampling of the experiments. The authors would also like to thank Charlotte Heynssens, Bhabananda Biswas and Tara Grosemans for help with the qPCR analysis.

## Data accessibility

All data of the experiment will be available after publication in the Integrated Marine Information System (IMIS) database (VLIZ):

http://www.vliz.be/en/imis?module=dataset&dasid=5109

# Supplementary information

**Appendix 1:** Weight loss of decaying algae over the different time moments among the different treatments.

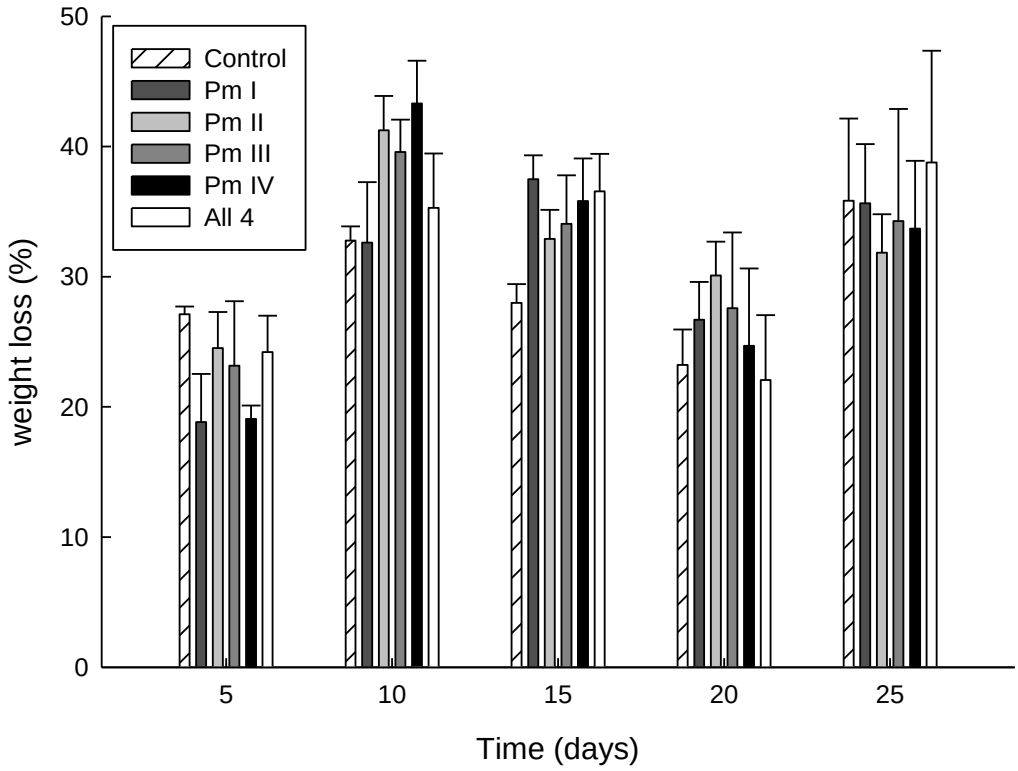



Figure S1: Weight loss (percentage ± SE) over the different time moments among the different treatments (n=100).

**Appendix 2**: detailed overview of the results of nematode densities

The number of adults was influenced by the three-way interaction of substrate, treatment and time (Fig. S2A and B, Table S1). Some species-specific significant differences in abundances of adults existed: Pm I showed higher abundances compared with Pm II and Pm III on the algae at day 5 and compared with Pm II, Pm IV and All4 at day 25 in the sediment, and for Pm I and All4 abundances were also higher than those of Pm IV. On the algae, Pm II showed higher adult abundances at day 20 compared with Pm I. Additionally on the algae, Pm III showed higher abundances compared with Pm I, Pm II and Pm IV at day 25 (Fig. S2A and B). Substrate had an effect on the nematode abundances: higher abundances were found for Pm I, Pm II and Pm III on the algae compared to the sediment on day 5. Abundances of nematodes increased most between day 5 and 10, and then remained nearly stable (Fig. S2). Pm III adults showed a different population increase on algae: abundances on day 5 only differed from day 25.

Abundances of juveniles were also influenced by the three-way interaction of substrate, treatment and time (Table S1, Fig. S2C and D). In the sediment, only at day 25 some differences were found: Pm I, Pm III and All4 showed higher abundances of juveniles compared with Pm II, and for Pm I and All4 also compared with Pm IV. On the algae, higher abundances were found for Pm II at day 10 compared with Pm III and Pm IV and at day 20 compared with Pm IV, for Pm I at day 15 compared with Pm II and for All4 at day 25 compared with Pm I and Pm II. Abundances of juveniles were lower at day 25 compared with day 20 for Pm I, Pm II and Pm III on the algae and only for Pm II in the sediment. Abundances of juveniles were higher in the sediment compared with the algae for all the treatments at certain time moments (at day 15 for Pm III, day 20 for Pm I, Pm II and All4 and day 25 for Pm I, Pm III, Pm IV and All4).

Table S1: Results of the linear mixed model on abundances of adults and juveniles

| Factor | Adults | | Juveniles | |
|---|---|---|---|---|
| | **F** | **P** | **F** | **P** |
| Substrate[1] | 78.11 | **<0.0001** | 0.29 | 0.59 |
| Time[2] | 193.99 | **<0.0001** | 88.66 | **<0.001** |
| Treatment[3] | 3.78 | **0.0074** | 7.77 | **<0.001** |
| Substrate*Time | 115.11 | **<0.001** | 19.89 | **<0.001** |
| Substrate* Treatment | 10.53 | **<0.001** | 1.57 | 0.19 |
| Time*Treatment | 9.50 | **<0.001** | 7.08 | **<0.001** |
| Substrate*Time*Treatment | 2.52 | **0.0039** | 7.17 | **<0.001** |

[1]algae, sediment; [2]day 5, 10, 15, 20 and 25; [3] Pm I, Pm II, Pm III, Pm IV and All4

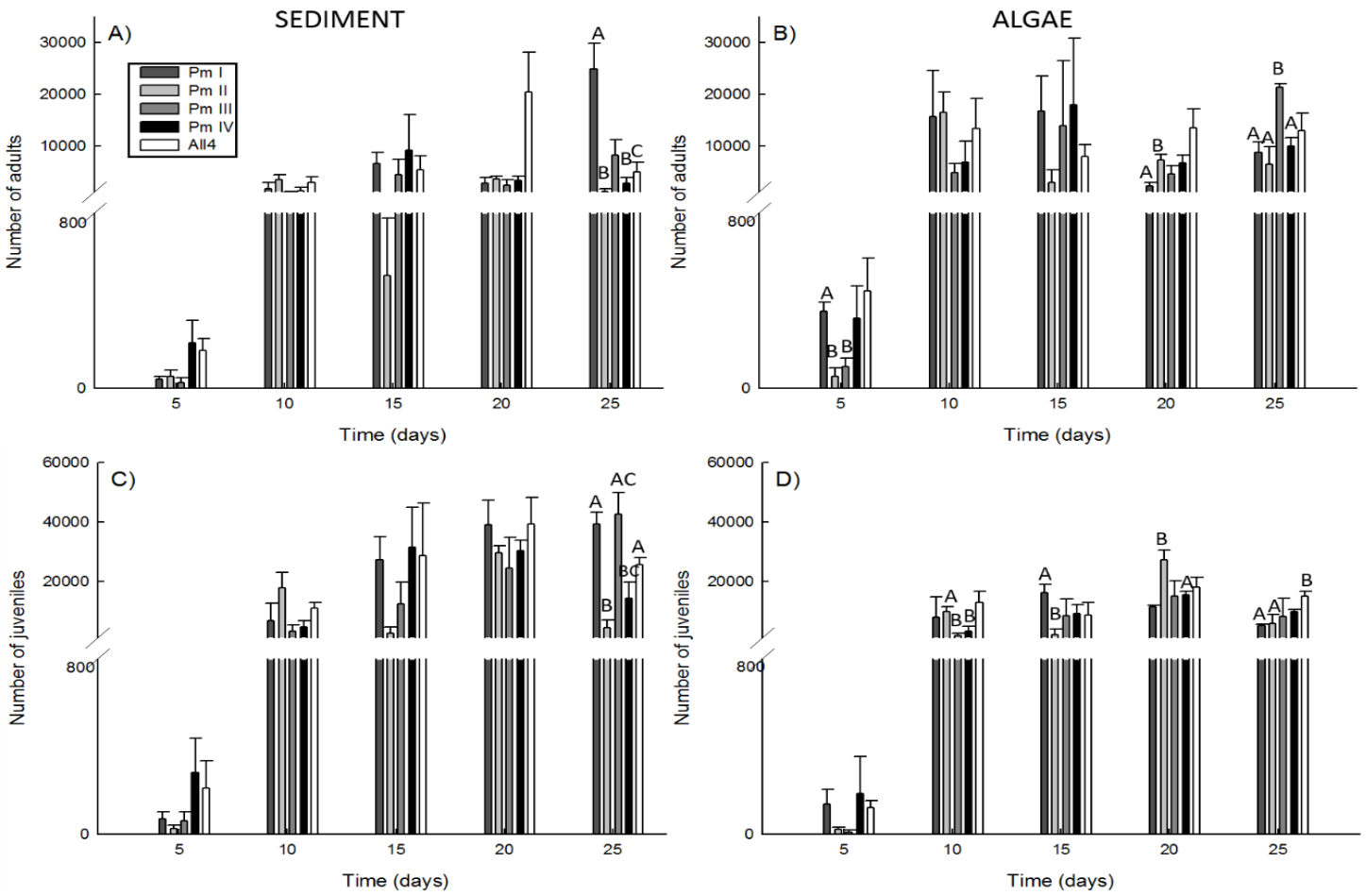



Figure S2.: Number of nematodes (mean + SE) on the algae and in the sediment at each time point for each of the different treatments: A + B adult abundances, C +D juvenile abundances. Letters show significant differences of the three-way interaction ($P<0.05$). Attention: an interval between 800 and 10000 is present and the scales of the y-axes differ before and after this interval. (n=100)

# CHAPTER IX

# GENERAL DISCUSSION AND FUTURE PERSPECTIVES

## Unravelling coexistence of cryptic species

Cryptic speciation already fascinates scientists for more than a hundred years, but studying the ecology and coexistence of cryptic species has only recently gained more interest (Molbo, et al., 2003; Sáez & Lozano, 2005; Vanelslander, et al., 2009; Derycke, et al., 2012; Fišer, et al., 2015). In this PhD thesis we used different experiments, the results of which we integrate in this general discussion with unpublished results of bachelor and master dissertations, to gain knowledge about cryptic species, their ecology and the mechanisms enabling their coexistence.

## How cryptic is cryptic diversity?

The different genetic lineages of *Litoditis "marina"* are indeed different species. Evidence for this was already found in genetic research: **molecular divergences** were found at three independent loci (COI, ITS, D2D3) (Derycke, et al., 2008a; Fonseca, et al., 2008). Moreover, there is reproductive isolation between the species (Fonseca, et al., 2008; Derycke, unpublished data). This **reproductive isolation** may originate from premating barriers, such as behavioral isolation (Futuyma, 2005). The cryptic *Litoditis* species showed a specific mate recognition system. Pheromones triggered a species-specific response for mating; Pm I and Pm IV nematodes were able to distinguish between specimens of their own and of different species (Heynssens, 2015). Moreover, also postmating barriers may exist. There is evidence of Pm I females copulating with Pm IV males, and although some eggs were present, they never formed embryos or juveniles (Fig. 1, Veltjen, 2012). According to the phylogenetic and biological species concepts, the cryptic species are thus separate species. Moreover, a multivariate morphometric analysis also showed that the cryptic species display at least some **morphological differences** (Fonseca, et al., 2008). The experiments in this PhD also show that the species differ in more aspects than first thought: differences in life-history traits (De Meester, et al., 2011 (chapter II), 2015b (chapter III) and 2015c (chapter IV)), carrying capacities (Pm III may reach higher densities than the other species, De Meester, et al., 2015b (chapter VII)), feeding ecology (Derycke, et al., 2016; chapter V), competitive abilities (De Meester, et al., 2011 (chapter II), 2015c (chapter IV) and 2015b (chapter VII)), dispersal strategies (De Meester, et al., 2012 (chapter VI) and 2015b (chapter VII) and even in their effect on the decomposition process (De Meester, et al., accepted; chapter VIII) were observed (overview in Table 1). The genetic distance between the species (Derycke, et al., 2005) was not always straightforwardly reflected in the ecological differences. Pm I and Pm

IV, the two most closely related species, for instance, exhibited the most pronounced differences in their respective roles in ecosystem functioning, while Pm I and Pm III, two species that are more genetically distinct, did not show any differences here.

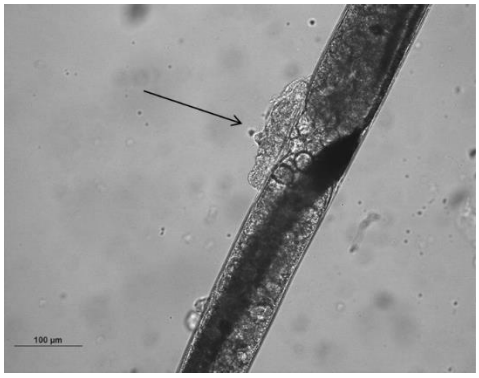



Figure 1: Picture of the vulva region of a Pm I female (Veltjen, 2012). Only Pm IV males were present in the same experimental microcosm as this female. Some sperm cells and 'eggs' are visible, however, no embryos and juveniles were formed, which points at the reproductive isolation of the species.

Table 1: Overview of the differences between the four cryptic species within the ranges of the tested abiotic conditions (standard conditions of 20°C, salinity of 25, unless otherwise noted). (*): interspecific competitive abilities were affected by species combination and abiotic conditions (salinity changes and fluctuating temperature).

| | | **Pm I** | **Pm II** | **Pm III** | **Pm IV** |
|---|---|---|---|---|---|
| *Life-history traits* | **Fecundity** | normal (3-4 offspring per female after 24h) | normal | high | normal |
| | **Reproductive strategy** | viviparous | oviparous | oviparous | dependent on salinity |
| | **Optimal salinity** | no distinct optimum | no distinct optimum | 15 | 15 |
| | **Optimal temperature** | no distinct optimum | 15°C | 25°C | 15°C |
| *Resource use* | **Interspecific differences in bacterial diet** | yes | not yet tested | yes | yes |
| *Competition* | **Interspecific competitive ability (*)** | strong | poor | intermediate – strong | poor |
| *Dispersal* | **Dispersal ability** | slow | intermediate | fast | intermediate |
| | **Main driver** | interspecific competition | time-dependent | density-dependent | density-dependent |
| *Ecosystem functioning* | **Effect on decomposition** | mostly inhibitory | inhibitory | mostly inhibitory | stimulatory |

## On the origin of cryptic species

But why don't the species of the *L. "marina"* species complex differ more in morphology? Predictions show that the species have diverged 16 MYA (Grosemans, et al., 2016), which renders the hypothesis of recent speciation, and by consequence a lack of sufficient time for morphological differentiation, unlikely. Speciation may also remain cryptic because of differentiation in sensory modalities, imperceptible for humans. An example are echolocation frequencies in bats (Jones, 1997) or imperceptible song differences in frogs or birds (Henry, 1994). For instance, differential echolocation frequencies may determine differences in resource use, and selection may be stronger for acoustic divergence than for morphological differences (Jones, 1997). Also differences in **pheromones** – which form a possible mechanism of mate recognition in *Litoditis "marina"* (see above) – may originate without morphological differentiation. This could be one of the explanations for cryptic speciation in the *L. "marina"* species complex. Another possibility is that morphological stasis is promoted and that species may continue to diverge genetically in the absence of morphological differentiation (Rocha-Olivares, et al., 2001). Such a strong stabilizing selection was already found to act on coccolithophorid phenotypes (Sáez, et al., 2003). Stabilizing selection will only play an important role if species communities are at equilibrium and in stable environments over long periods of time (Parsons, 1994; Lieberman & Dudgeon, 1996). *L. "marina"* occurs in very fluctuating habitats, but these are stable when viewed over longer time periods (Southwood, 1977). If the morphology of the species is adapted to the fluctuations of the tidal environment, stabilizing selection may act on it.

The most plausible explanation for the lack of obvious morphological differentiation among *L. "marina"* species is, however, that speciation was accompanied by morphological differences and that diagnostic characters could be found when cryptic species would be studied in more detail (Pfenninger & Schwenk, 2007). A closer look on cryptic species of liverworts allowed to discriminate them by differences in the coarse appendages at the leaf margins and the papillose leaves (Feldberg, et al., 2004). In rotifers, cryptic species were morphologically differentiated by the trophi, the surface structure and the form of diapausing eggs (Schröder & Walsh, 2007). For some fungi, cryptic species have even been formally described after the discovery of previously unnoticed morphological differences (Alves, et al., 2008). The apparent absence of morphological differences may mostly be the result of overly **conservative systematics** (Klautau, et al., 1999). Some taxa (such as nematodes and sponges) are characterized by only few morphological characters, which further complicates

the distinction of nearly identical species (Bickford, et al., 2007). Moreover, morphological variation may also be high within a species (Kiontke & Fitch, 2010; Fonderie, et al., 2013), which renders correct species identification even more difficult. A more detailed study on the cryptic species complex of *L. "marina"* may reveal **more (easy to distinguish) morphological differences.** For instance, scanning-electron microscopy pictures have revealed the presence of a cuticular depression around the vulva of Pm III females, which was absent in the females from the other species (Fig. 2). This contributes to the hypothesis that cryptic speciation may have been accompanied by at least some morphological differences.

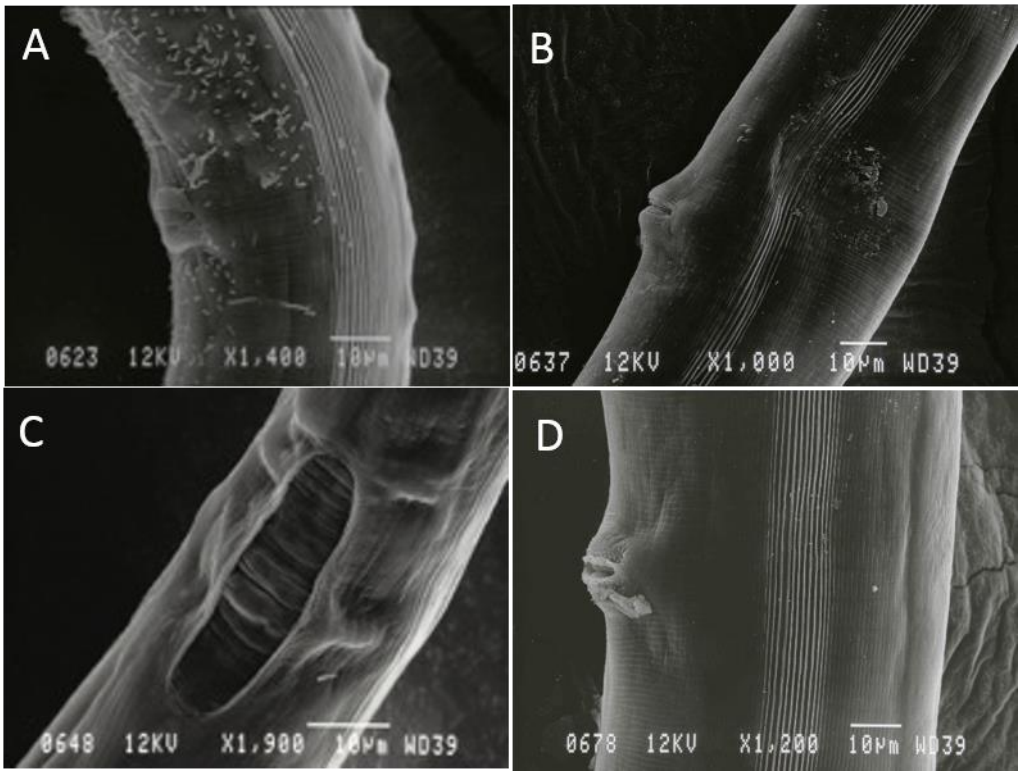



Figure 2: Scanning-electron microscopy pictures of the vulva region of a A) Pm I, B) Pm II, C) Pm III and D) Pm IV female. A cuticular depression around the vulva of the Pm III female is visible (pictures provided by Derycke S.)

## Strong competition between the species: intransitive interactions and environmental dependency

Darwin (1859) stated that all species are engaged in a competitive '*Struggle for existence'*. This was seen as a struggle for food to support growth, life and reproduction. Species that are very similar will struggle more because they need the same type of resources, and only the fittest species will survive. Ecological differences between the species exist (see table 1); nevertheless our experiments (De Meester, et al., 2011 (chapter II), 2015c (chapter IV) and 2015b (chapter VII)) show that competition existed between the cryptic species of *L. "marina"*. However, it was impossible to detect one ultimate competitively superior species in all experiments (see Fig. 3 for an overview of interspecific interactions for Pm I). The cryptic species can thus not easily be ordered hierarchically, probably because a **competitively intransitive network** exists. Such network is characterized by at least one **competitive loop**. In a competitive loop, species A, for instance, is competitively superior over species B, which is competitively superior over species C, which is in turn competitively superior over species A. In the *Litoditis "marina"* complex we have some evidence for at least one loop: Pm I was competitively superior over Pm III (De Meester, unpublished data), Pm III over Pm II (Hugo, 2012), and there is some evidence that Pm II benefitted from the presence of Pm I and may even be competitively superior over Pm I (Fig. 4, Hugo, 2012). Future competition experiments on all pair-wise combinations with the four cryptic species may highlight even more loops, or may reveal an intermediate level of intransitivity, with both loops and hierarchical orders (Laird & Schamp, 2006). Such loops make it extremely difficult to predict the outcome of competition when the number of species changes. For instance, based on the experiment with the four species in closed microcosms (De Meester, et al., 2011; Chapter II), we predicted that Pm I would be the strongest competitor, but in pairwise combinations with other species this was not always observed (Fig. 3). Such intransitive networks seem to be quite common in nature and occur in a broad range of species (overview in Laird & Schamp, 2008).

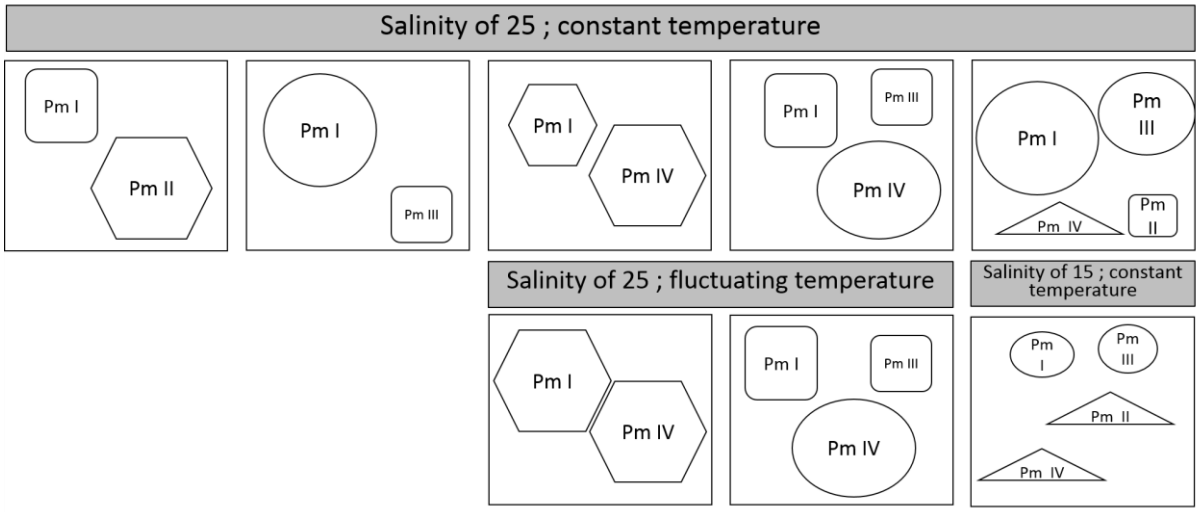



Figure 3: Overview of the different competitive abilities of Pm I when combined with different species and/or when subjected to differential abiotic conditions. A circle illustrates a competitively superior species (has a negative impact on one of the other species, but is not negatively influenced itself), a square illustrates a competitively inferior species (abundances are negatively influenced by the presence of another species), a hexagon is used for species that are not (or even positive) affected by the presence of other species, a triangle illustrates that this species was competitively excluded. The size of the shapes illustrates the relative dominance of the species by the end of the experiment. The data was collected from De Meester, et al. (2011 (chapter II), 2015c (chapter IV), unpublished data) and Hugo (2012).

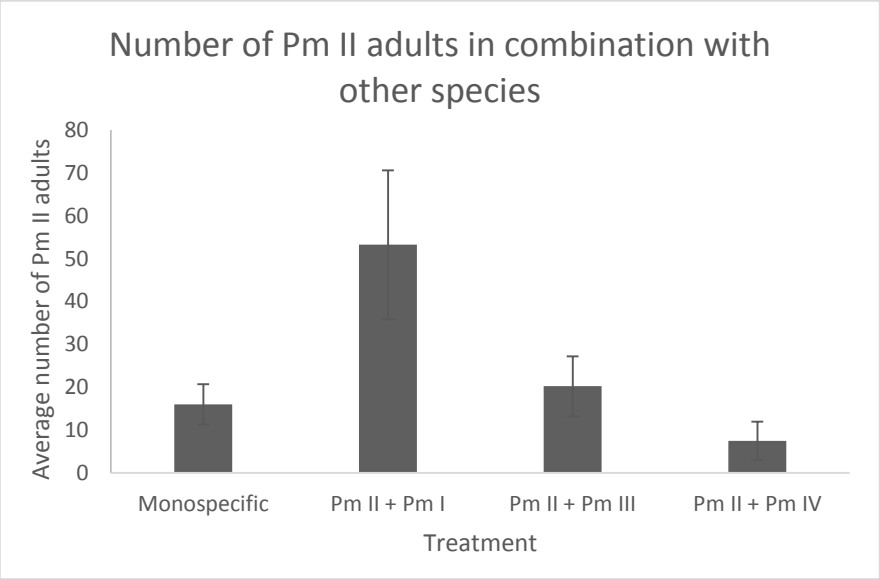



Figure 4: Average number of Pm II adults after ten days (inoculation of 5 females and 5 males at the start of the experiment) in monospecific conditions and in combination with other species (resp. Pm I, Pm III and Pm IV). Pm II showed higher adult abundances when Pm I was present. (Hugo, 2012).

Intransitive competition can originate from both exploitation and interference competition (Laird & Schamp, 2006). Exploitation competition is competition in which organisms will compete for food, space or other necessary resources by using the resources before the other species can do so. In interference competition one species interferes directly with the ability of another species to obtain resources. Three possible mechanisms can lead to intransitive competition: 1) if one species competes best for some resources but is limited by another different resource (Huisman & Weissing, 1999) and the other species differ in these abilities to compete; 2) if the two types of competition act simultaneously but the competitive abilities of the interference competition differ from these of the exploitation competition (Czárán, et al., 2002); or 3) if purely interference competition exists (interference behavior differs depending on the other species) (Czárán, et al., 2002). From our experiments, we do not know the exact nature of the competition, but **exploitation competition for food** may play a role as species started to disperse sooner when food was absent (De Meester, et al., 2012; chapter VI). Nevertheless, if food was present, density-dependent dispersal existed, regardless the amount of food (De Meester, et al, 2015b, chapter VII) indicating that competition for **other resources** (for instance space) or **interference competition by**

**chemical repulsion** may also occur (Huettel, 1986; Chandler & Fleeger, 1987). Mucus trails produced by the nematodes (and frequently observed in all cryptic species of *L. "marina"*) may repel other species or may even inhibit growth of other species, although evidence in marine nematodes is still absent.

Predicting the outcome of competition becomes even more complex when **abiotic conditions** change, as this **affects the strength or type of interactions between the species** (De Meester, et al., 2011 (chapter II) and 2015c (chapter IV)). Environmental conditions under which the competition occurs can partly determine the outcome of interspecific interactions (Fujii, 1968) by the presence of differential physiological tolerances to abiotic factors (Dunson & Travis, 1991) or by directly influencing interference behaviour (Amarasekare, 2002). Within an intransitive network, this may lead to unexpected shifts in the interactions. For instance, in the *L. "marina"* cryptic species complex competition appeared to be more severe when constant temperature changed to fluctuating temperature, and a shift from commensalism to a sort of mutualism was observed in experiments with two species (De Meester, et al., 2015c ; chapter IV). This cannot readily be correlated with the species' respective performance at these specific abiotic conditions, which leads us to the suggestion that **competition may be directly influenced by abiotic factors**. An even more special case is the shift from **contest to scramble competition** when salinity changes (De Meester, et al., 2011; chapter II). Whereas at a higher salinity some species were able to compete more for sources than others (contest competition), at a lower salinity all cryptic species competed equally for the resources and none was initially able to fully meet its needs (scramble competition). In the latter case all species may go extinct, or some may be able to survive. On the one hand this survival may be the result of random processes: if by chance some species die off earlier than others, the remaining species may be able to recover. On the other hand, if species have life-history traits that are **better adapted to the specific abiotic conditions**, they can become 'the survivor' of the scramble competition, despite the fact that they also suffer from that competition (Gilpin, 1974; Lale & Vidal, 2001). This last explanation may be the reason why Pm III survived when all four cryptic species were placed together at the lower salinity (De Meester, et al., 2011; chapter II): Pm III may suffer from the competition at low salinity, but because it exhibits a better population development at a lower salinity (De Meester, et al., 2015a; chapter III), it may survive the competition and become abundant. This cannot be the explanation for the survival of Pm I, as no higher population development was found at the lower salinity, so **randomness** may also be

important. Pm I may suffer from the competition at lower salinity, but may still be competitively superior over the other species. When random factors lead to some population growth of Pm I, Pm I may easily overwhelm the other species and survive the scramble competition. The fact that abiotic conditions change the type and strength of interactions indicates that competitive abilities of cryptic species may change when environmental factors change (Amarasekare, et al., 2004). This is also quite common in nature (overview in Dunson & Travis, 1991), nevertheless most studies still focus only on the role of abiotic factors on one species (e.g. Brakefield & Kesbeke, 1997; Pétavy, et al., 2001; Colinet, et al., 2007).

Abiotic changes, changes in population densities or species composition can drive communities from one stable state to another (Beisner, et al.,2003). Nevertheless, if environmental perturbations are common, communities may never reach such a stable state, because the change in the environment may lead to a change in the outcome of interspecific interactions. In our decomposition experiment (De Meester, et al., accepted; chapter VIII) - which most mimics the natural conditions of the species- community composition was still changing, possibly as the result of the continued decomposition process of the algae. This indicates that a stable state was not reached and coexistence and interspecific interactions of the species are found at a **non-equilibrium state**.

Not only environmental conditions may be important for the outcome of competitive interactions. Shifts between types of interactions can also occur between **different life stages** (Soliveres, et al., 2010), which was also found for *Litoditis "marina"*: adults of Pm I and Pm III were not influenced by the presence of each other in combination with Pm IV, but juveniles were influenced in a negative way. This can be a consequence of the fact that juveniles may be more sensitive to stress than adults (Martinez, et al., 2012), or of an effect on the fecundicity of the adults. These experiments were all conducted in closed environments. Adding **spatial heterogeneity** to these experiments (De Meester, et al., 2015b; chapter VII) also changed the outcome of the competition (see further).

Based on Darwin's theory we can expect that competition will be the highest between Pm I and Pm IV, as these are the two most closely related species (Derycke, et al., 2005). Nevertheless these species were able to coexist in some experiments (De Meester, et al., 2015c; chapter IV). All these results show that competition between cryptic species is very common and that some mechanisms are necessary to achieve coexistence.

## Towards a coexistence framework.

Different paradigms that explains coexistence exist (Leibold & McPeek, 2006) (for an overview of the different paradigms, see general introduction (chapter I) and Table 2). In the typical ephemeral habitat where *L. "marina"* lives, coexistence on a local scale (one piece of alga) and regional scale (different algae on one location) can both occur.

**Niche differentiation** clearly occurs in the studied cryptic species complex, and competition between cryptic species may be weakened by this ecological segregation. The microbiomes of Pm I, Pm II and Pm III partly differed and when offering different bacterial food mixes, Pm I and Pm III exhibited different resource selectivity (Derycke, et al., 2016; chapter V). **Food partitioning** has already been demonstrated to be an important way to achieve coexistence in other cryptic species complexes: diet analysis in bat species revealed that they share the same dominant food source but can complement this with different food sources (Razgour, et al., 2011). Such fine-scale differences in resource use, as also found for the *L. "marina"* complex, may lead to coexistence in a fluctuating environment if three assumptions are fulfilled: 1) a stage buffered from competition exists, 2) differences in life history exist depending on (a)biotic conditions, and 3) a heterogeneous competitive environment exists (Chesson, 2000). All three aspects can be validated for the *L. "marina"* cryptic species complex: 1) *Litoditis "marina"* can produce a **dauer stage under harsh conditions** (Derycke, et al., 2008b) that will develop further only when conditions turn favorable again; 2) different life histories between the species exist depending on environmental conditions: we can expect that **fluctuations** in bacterial composition may influence their life histories, but species still show a **high overlap between their optimal food conditions**. Temporal changes may favor one species at a certain time, but another species at another time, and coexistence dynamics may occur. Moreover, differences in food resource may not be the only niche diversification mechanism here. We found that species differ in their salinity and temperature preferences (De Meester, et al., 2015a; chapter III), despite the fact that here too, considerable overlap between the preferences/tolerances of the cryptic species exists. In absence of interspecific interactions, we expected Pm II to dominate at lower temperatures and Pm III at higher temperatures (Fig.5). Species are, however, also able to survive at the other temperatures and population growth can increase rapidly when temperature conditions turn beneficial. These differences in abiotic tolerances/preferences may lead to coexistence and may even be indirectly linked with food diversification. Abiotic niche differentiation has also been invoked as a likely explanation for the coexistence of

cryptic species of rotifers, sea urchins and nematodes (Palumbi & Metz, 1991; Ortells, et al., 2003; Montero-Pau, et al., 2011; Van Campenhout, et al., 2014) And 3) **competition differs when environmental changes occur** (De Meester, et al., 2011 (chapter II) and 2015c (chapter IV)) (see above).

Ecological character displacement may be the mechanism behind this niche differentiation: species may accentuate some morphological, ecological, behavioral or physiological characters when they go in interaction with other species (Brown & Wilson, 1956), while these differences may be minimalized when they occur alone. Such niche differentiation, can explain coexistence on a local scale (one piece of algae) if temporal fluctuations are present. In addition, even spatial heterogeneity may be important at such a small scale: we never obtained Pm I and Pm IV from the same algal thallus, but they were detected as soon as we included the floating bladders of the algae (Van Damme, 2015).

Nevertheless, in **fast changing and disappearing habitats** – such as the macroalgae inhabited by *L. "marina"* – **dispersal** to new patches is extremely important and not only local but also **regional coexistence** has to be achieved because species will have to disperse before the natal sites turn unfavorable (Snyder & Chesson, 2003). The above mentioned niche differentiation, combined with the fact that active dispersal does occur in the cryptic species complex (De Meester, et al., 2012; chapter VI) leads us to **spatial storage** as the most plausible explanation for regional coexistence in our cryptic species complex. Abiotic and biotic conditions can differ between two nearby (algae) patches, and if species are able to disperse to a more preferred patch, regional coexistence may exist.

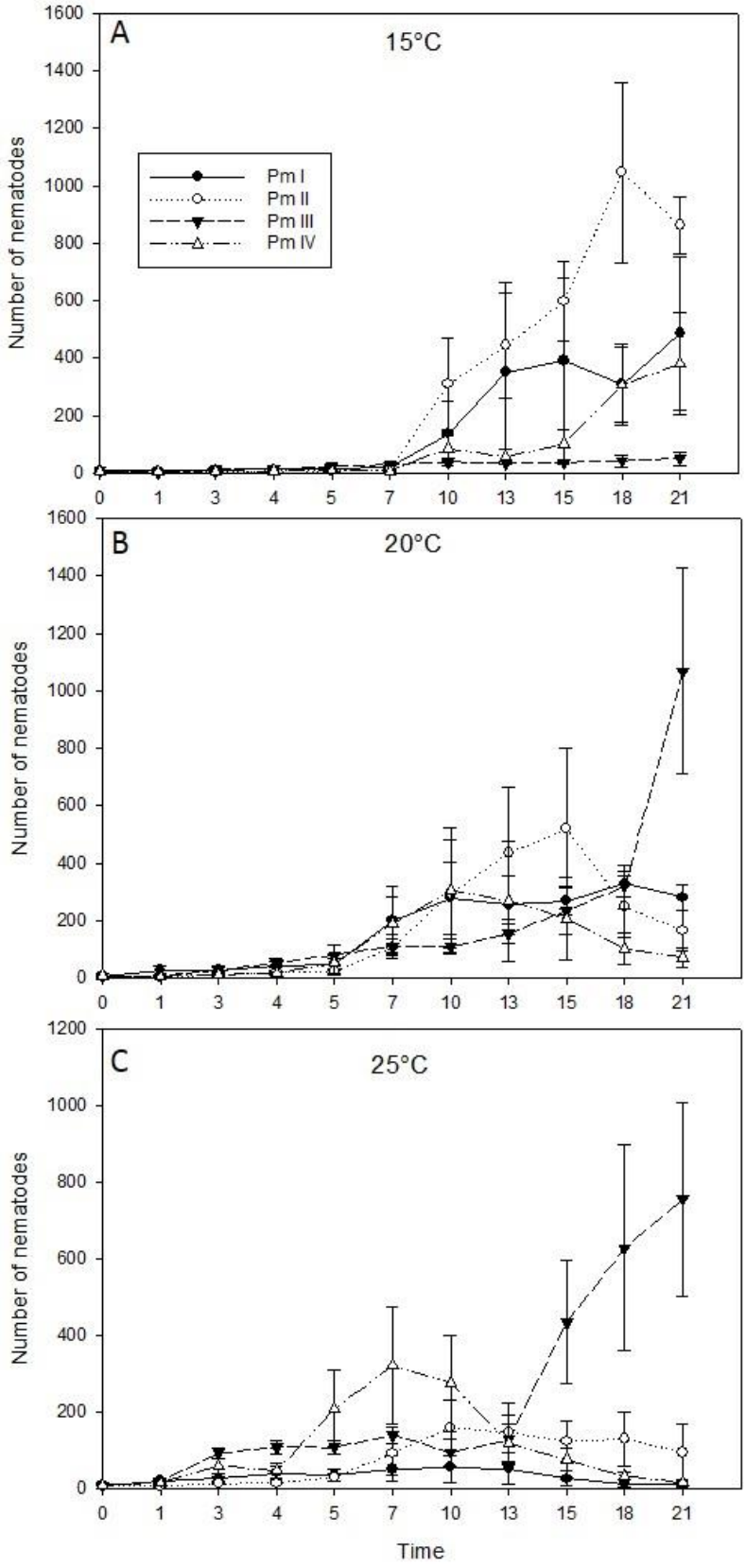



Figure 5: Population development over time (mean ± SE) for the four cryptic species of *L. "marina"* at three different temperature treatments: a) 15°C, b) 20°C and c) 25°C (adapted from chapter III).

But can we exclude all other metacommunity paradigms? **Colonization-competition trade-offs were not observed** within our cryptic species complex (De Meester, et al., 2015b; chapter VII), which renders the patch-dynamics hypothesis very unlikely. Nevertheless, species-specific dispersal costs may exist (see further) and may still result in a colonization-competition trade-off: if species with a lower dispersal cost, are able to allocate more energy to reproduction (or competition), they may have some advantages over species with higher dispersal costs. This can be the case for Pm II: although it was not the fastest disperser (De Meester, et al., 2012; chapter VI), we have some indication that dispersal goes at a lower cost compared with other species (see further). Pm II may be able to allocate more energy to reproduction or competition compared with the other species and this may explain the better performance of Pm II in combined experiments with dispersal opportunities compared with closed experiments. Nevertheless, even if such trade-offs exist, spatial heterogeneity may easily overwhelm them (Levine & Rees, 2002). The mass-effect paradigm may be plausible as it occurs in a heterogeneous ecological and competitive environment, but it seems very unlikely as sources and sinks may change continuously (due to the temporal existence of macroalgae patches) and **dispersal in meiofauna is mostly limited** (see further).

At first sight, **neutral dynamics** also seem unlikely because differences between all species were found. Nevertheless, **competitive intransitivity** (see above) may represent **an important mechanism for ecological equivalence** because each species outcompetes, and is outcompeted by, an equal number of competitors. Intermediate levels of intransitivity promote species coexistence by slowing the process of competitive exclusion, and random processes may become important (Laird & Schamp, 2006, 2008). This indicates that even if differences exist between the species, neutral dynamics may still play a role. Moreover, Pm I and Pm IV – the two most closely related species – were very similar in their reproduction strategy, morphology and most ecological characteristics, and they had no negative influence on each other when they occurred together (see fig. 3, third column). In addition, intraspecific and interspecific competition had a similar effect on dispersal of Pm IV (De Meester, et al., 2015b; chapter VII). If species differences are very minimal, and/or intraspecific competition is as high as interspecific competition, species coexistence will be dependent on neutral processes. As a result, a combination of different mechanisms to explain coexistence cannot be excluded.

Table 2: the different coexistence paradigms and their minimal conditions. The final row is a conclusion of the observed data for the cryptic species complex of *Litoditis "marina"*.

| | | **ABIOTIC ENVIRONMENT** | **COMPETITIVE ENVIRONMENT** | **ECOLOGICAL DIFFERENCES** | **DISPERSAL DIFFERENCES** | **POPULATION DYNAMICS VS. COLONIZATION-EXTINCTION DYNAMICS** |
|---|---|---|---|---|---|---|
| **LOCAL COEXISTENCE** | Niche differentiation | Homogeneous | No competition between the species | Present | Not applicable | Not applicable |
| **REGIONAL COEXISTENCE** | Patch-dynamics | Homogeneous (patches do not differ in suitability) | Homogeneous | Present | Present | Different time scales |
| | Spatial storage | Heterogeneous, temporal or spatial | Heterogeneous | Present | Absent or Present | Different time scales |
| | Mass-effect | Heterogeneous, spatial | Heterogeneous | Present | Absent | At same time scale |
| | Neutral dynamics | Homogeneous | Homogeneous | Absent | Absent | Affected by random processes |
| **MODEL SPECIES** | *L. "marina"* cryptic species complex | Heterogeneous, temporal and spatial | Heterogeneous | Present | Present | ? |

## Dispersal in meiofauna: paradox or essential?

In spatially heterogeneous environments, dispersal is extremely important (Snyder & Chesson, 2003) to be able to move away from (temporarily) unfavourable conditions. Although most meiofaunal species are widely distributed, **their mechanisms of dispersal are still under discussion** (Boeckner, et al., 2009). Passive dispersal is recognized as one of the main mechanisms for meiofauna to reach new habitats. Nematodes are able to **passively disperse with the water flow** following erosion from sediments or through **rafting on algae** (Thiel & Gutow, 2005). The water-flow dispersal may also help to explain widespread distributions of small species by stepping-stone dispersal (Gandon & Rousset, 1999): cycles of resuspension, transport and settlement may be repeated several times (Thomas & Lana, 2011). Moreover **dispersal through the air** may also occur for littoral nematodes (Buys, 2014); this may be possible just by the wind, but more evidence points to hitchhiking on flying insects, and was observed for *L. "marina"* (Fig. 6, Buys, 2014). **Active dispersal** is considered to be less common (Boeckner, et al., 2009). Nevertheless, nematodes may migrate laterally through sediments or swim distances up to 1m (Schratzberger, et al., 2004; Ullberg, 2004; Gallucci, et al., 2008; Thomas & Lana, 2011). In the cryptic species complex of *L. "marina"*, active dispersal and attraction towards food were found, suggesting that active dispersal may also be important (De Meester, et al., 2012 (chapter VI) and 2015b (chapter VII)). Such **small-scale active migration may be useful to avoid competition**, for instance by moving towards new algal patches or floating bladders or receptacles on the same patch.

Moreover, nematodes may also be able to **partly control the passive dispersal** process (Armonies, 1988; Ullberg & Olafsson, 2003; Schratzberger, et al., 2004; Guilini, et al., 2011; Van Daele, 2014): dispersal is a three-factorial process that consists of – besides the **transience** process – a **departure and settlement** stage (Fig. 7, Clobert, et al., 2009), which nematodes can partly control themselves. Previous studies (Jensen, 1981; Armonies, 1988) already showed that nematodes are able to actively enter the water column. This was also proven for all four cryptic species *of L. "marina"* in annular flume experiments: they were able to enter the water column without the presence of a water flow (Van Daele, 2014). This indicates that they can control their suspension into the water column, which may facilitate both small-scale active dispersal as well as larger-scale passive dispersal (Chandler & Fleeger, 1983). Nematodes are also able to at least partly control their settlement (Ullberg & Olafsson, 2003; Schratzberger, et al., 2004; Guilini, et al., 2011; Lins, et al., 2013;

Mevenkamp, et al., 2016), in which they actively choose a preferred type of substrate. In the *L. "marina"* species complex, such a specific settlement was also found (Fig. 8; Van Daele, 2014): most cryptic species preferred to settle on patches with algae and not on patches with only sediment. But will dispersal be that important in the *L. "marina"* species complex? Yes! As a **patch inhabited by the species can rapidly change or even disappear**, it may be extremely important to be able to disperse. Algal patches that are not suitable anymore – due to space or food restrictions, predation or competition – will be left by the nematodes. *L. "marina"* is able to partially control its departure and may be able to choose when to leave (De Meester, et al., 2012 (chapter VI); Van Daele, 2014), which may increase the probability of successful dispersal.

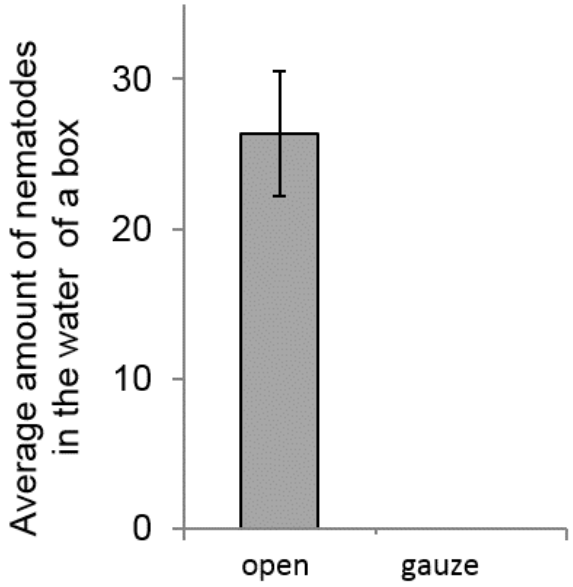



Figure 6: Average amount of nematodes (± SE) of a field experiment, dispersed via air after one week. The field experiment consisted of wooden boxes with defaunated algae. The boxes were randomly placed at ca. 1.75m height at the Paulina salt marsh (The Netherlands) in autumn of 2013. All the boxes (25 x 23 x 23cm) possessed a shelter to avoid freshwater from precipitation entering the boxes. Half of the boxes were open (testing dispersal by wind as well as by hitchhiking on insects), while the other half were covered with a gauze of 200µm, to prevent insects from entering (only wind dispersal possible). This experiment showed that dispersal through the air is common and is the result of hitchhiking on insects. Moreover, identification of the collected nematodes revealed that *L. "marina"* was present in the open boxes (Buys, 2014).

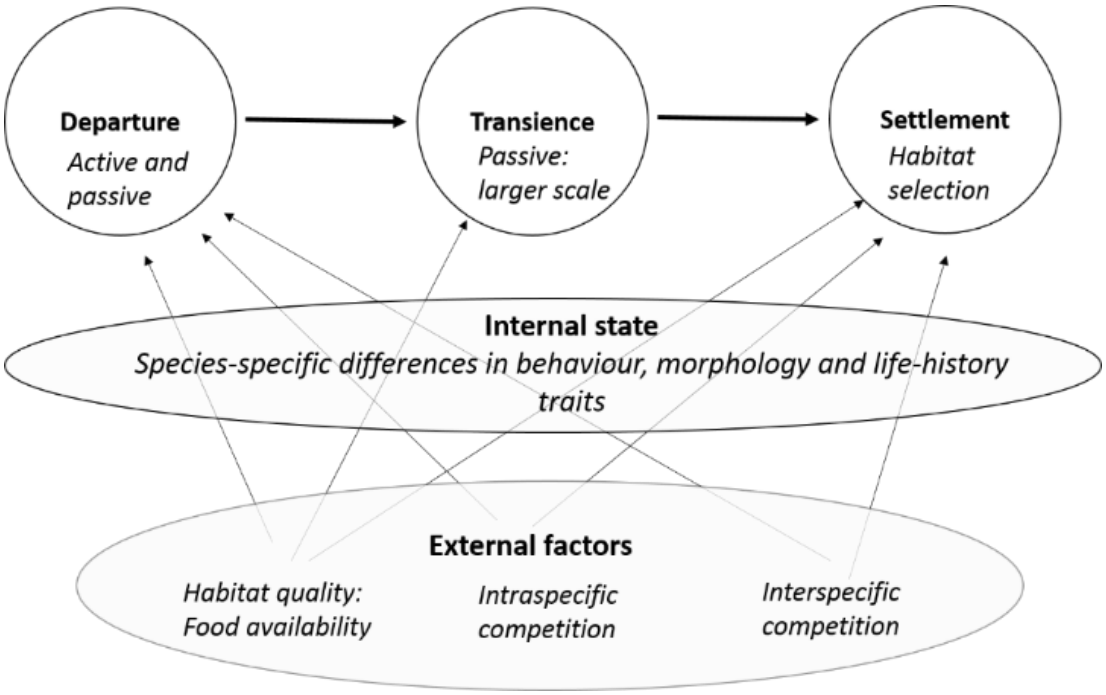



Figure 7: Conceptual framework to investigate individual variation in dispersal (adapted from Clobert, et al., 2009) applied to the *Litoditis "marina"* cryptic species complex. Relationships between the three dispersal phases (departure, transience and settlement), phenotype-dependent dispersal (i.e. dependence on internal state) and condition-dependent dispersal (i.e. dependence on external factors) are illustrated by arrows. Departure and settlement can be partly active processes, transience mostly passive. Factors influencing internal state and external factors are in italics.

Figure 8: Experimental set-up of the settling experiment for *Litoditis "marina"*. Top view of one of the settling containers with 2 sediment quadrants (left & right) and 2 algae quadrants (top & bottom), separated by 4 metal separation plates (14 x 4 cm). All experiments were conducted with one species (Pm I, II, III or IV). Around 1000 nematodes per species were released into the water. After 48h the biggest proportion of nematodes was found in the algae quadrants for Pm II; III and IV. Pm I did not show any preference (Van Daele, 2014).

In a heterogeneous habitat, where patches vary spatially and temporally, differential dispersal strategies are likely to evolve among closely related species (McPeek & Holt, 1992). Dispersal can be influenced by internal and external factors during all phases. In nematodes, dispersal is influenced by the morphology and swimming ability of the species (Thomas & Lana, 2011). Differences in dispersal were also found in the *L. "marina"* complex, but movement analysis (Fig. 9, Monteiro & De Meester, unpublished) and flow experiments (Van Daele, 2014) did not show any differences between the species, suggesting that the **differences between the species** are situated in **other stages of dispersal than the transience process.** Dispersal triggers and time until dispersal differed between the species (De Meester, et al., 2012 (chapter VI) and 2015b (chapter VII)) and species-specific settlement was also found (Van Daele, 2014). An overview of the different stages and factors influencing dispersal can be found in fig. 7. These dispersal differences may be the consequence of different **dispersal costs** between the species. Pm III showed to be a typical

r-strategist: it dispersed very fast, long before the carrying capacity of the environment was reached. The organism that first dispersed was always one gravid female, which produced many offspring in the dispersal plate. Costs for dispersal in Pm III are expecting to be low, with Pm III dispersing already long before the patch turns unfavourable. For both Pm I and Pm IV dispersal costs are expected to be higher: these species dispersed slow if conditions were favourable, but showed faster dispersal if intraspecific and interspecific interactions occurred. At the first dispersal event, only Pm I females dispersed, which may point out that dispersal costs are lower for the females compared with the males in Pm I. Pm II showed an unconditional dispersal, which may indicate a low cost to disperse (McPeek & Holt, 1992). Despite the fact that most coexistence can occur without differences in dispersal strategies, **sufficiently large dispersal differences between species may produce stable coexistence** (Aiken & Navarrete, 2014). Differences in dispersal costs may result in differences in energy allocation for competition and can result in coexistence (see above). In addition, differences in dispersal abilities (for instance time until dispersal) can lead to a competitively inferior species able to persist in a certain environment by occupying a dispersal niche that differs from that of a superior species (Aiken & Navarrete, 2014). Pm III was the fastest disperser in our experiments and may have a completely different dispersal niche than a slow disperser like Pm I. When individuals of Pm III leave a patch, where Pm I was already present, this can result in open spaces, which can be easily occupied by more individuals of Pm I. By the time individuals of Pm I start to disperse and arrive at the patch where Pm III already dispersed to, some individuals of Pm III may already leave that patch again. This creates a dynamic environment with species arriving and leaving patches at different time moments and thus leading to a temporally form of coexistence. This dispersal-based mechanism can be combined with other paradigms, such as the spatial storage effect, which was already proposed as one of the possible paradigms leading to coexistence in our model system (see above).

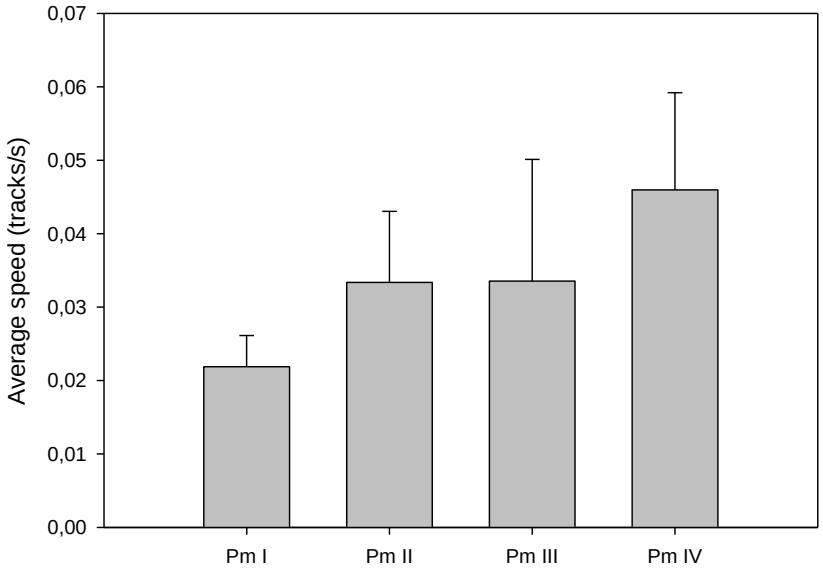



Figure 9: Average speed (± SE) of the four cryptic species. Ten nematodes per species were individually recorded for one minute and number of tracks per second was measured with the WormTrack plug-in (Fiji (Schindelin, et al., 2012)). No differences were found with a Kruskal-Wallis test between the cryptic species (Monteiro & De Meester, unpublished).

Moreover, dispersal may have been extremely important in the past to achieve the sympatric occurrence of the cryptic species. Allopatric speciation is considered the most common speciation mechanism in marine species (Wilke & Pfenninger, 2002). This was also implicitly assumed for *L. "marina"* by Derycke, et al. (2008b). Coexistence of the cryptic species was explained by long-distance dispersal events leading to sympatry among species.

## Cryptic species matter

Conservation biologists agree that every ecosystem and species is unique to some degree. As a consequence they **aim to avoid the extinction of any species** and confirm that each species and ecosystem requires a unique approach to management (Lindenmayer & Hunter, 2010). This is in contrast with the redundancy hypothesis (Walker, 1992), which states that ecologically similar species may compensate for each other when a species goes extinct and some species are thus less important for an ecosystem. Conservation management mostly starts with the question: 'what do we want to conserve?' (Fig. 10). Whether the answer is a species, an ecosystem service or a habitat, **cryptic species immediately complicate** this question. Conservation biologists base most of their activities on species **without recognizing the limitations of the taxonomic information** they use (Rojas, 1992). In the last two decades, the use of an **evolutionary significant unit** (ESU) already stressed this problem (Moritz, 1994). An ESU is genetically separated from other populations, and contributes to ecological and/or genetic diversity found within a species. In most cases, an ESU is thus identified by criteria of the phylogenetic species concept (Vogler & Desalle, 1994; Hey, et al., 2003) and used in conservation biology because they are indicators of evolutionary processes.

Is it important to conserve each cryptic species? Until now, research did **not include cryptic biodiversity into ecosystem services**, but this PhD thesis shows that each cryptic species may differ substantially and as a consequence may contribute differentially to the decomposition process (De Meester, et al., accepted; chapter VIII). Pm IV had a more stimulatory effect on the enzymatic activities, involved in the decomposition process, compared with the other cryptic species. If these species differences are big enough and as a consequence the loss of one cryptic species may cause losses in ecosystem functioning, it may be extremely important to **conserve all cryptic species.** This may be revealed by additional experiments with different numbers of species and their influence on the decomposition process.

The status of current habitats (step 2 in Fig. 10) is mostly quantified by the use of some key species (Mills, et al., 1993), for instance for **monitoring pollution** (Resh & Unzicker, 1975). In this step, cryptic species may also bias results. Cryptic species of *L. "marina"* showed species-specific tolerances towards pollutants (Van Butsel, 2014), which was also found for copepods (Rocha-Olivares, et al., 2004). Neglecting cryptic diversity may thus lead to wrong conclusions about the impact of pollutants and ecosystem health.

In addition, the effect of (future) climate change is often incorporated in species and habitat conservation strategies. Even if there are no differential responses to **climate change** between cryptic species, our study shows that there may be **consequences on the interactions between the species**. Decreases in salinity (for instance caused by water level rise) or decreasing amplitudes of daily temperature fluctuations (De Meester, et al., 2011 (chapter II) and De Meester, et al., 2015c (chapter IV)) may change the interactions between the cryptic species (see above), which may even translate into effects on ecosystem services.

It is thus no surprise that cryptic species should be involved in conservation biology. A **management plan** starts with two important points (see fig. 10): the identification of what you want to conserve and the current state of what you want to confirm. If information in these steps is incomplete, all the strategies to achieve these goals may be unsuitable. This fact combined with the knowledge that cryptic diversity is found in almost all biogeographical regions and taxa (Pfenninger & Schwenk, 2007) and new cryptic species are still discovered at a high rate, makes us propose to investigate cryptic diversity in the species or habitat of interest. We cannot predict if ecological differences will exist between other cryptic species, but this PhD thesis shows that cryptic species can differ more than we expect, and this can have consequences on ecosystem functioning. Until proved otherwise, we advise to include all cryptic species in conservation biology. Moreover, cryptic species are also found within key species for ecosystem functioning and pollution monitoring. As a consequence, we also advise that **genetic studies should be conducted on key species**. If cryptic species are discovered within a key species, ecological studies to investigate important differences between the cryptic species are necessary.

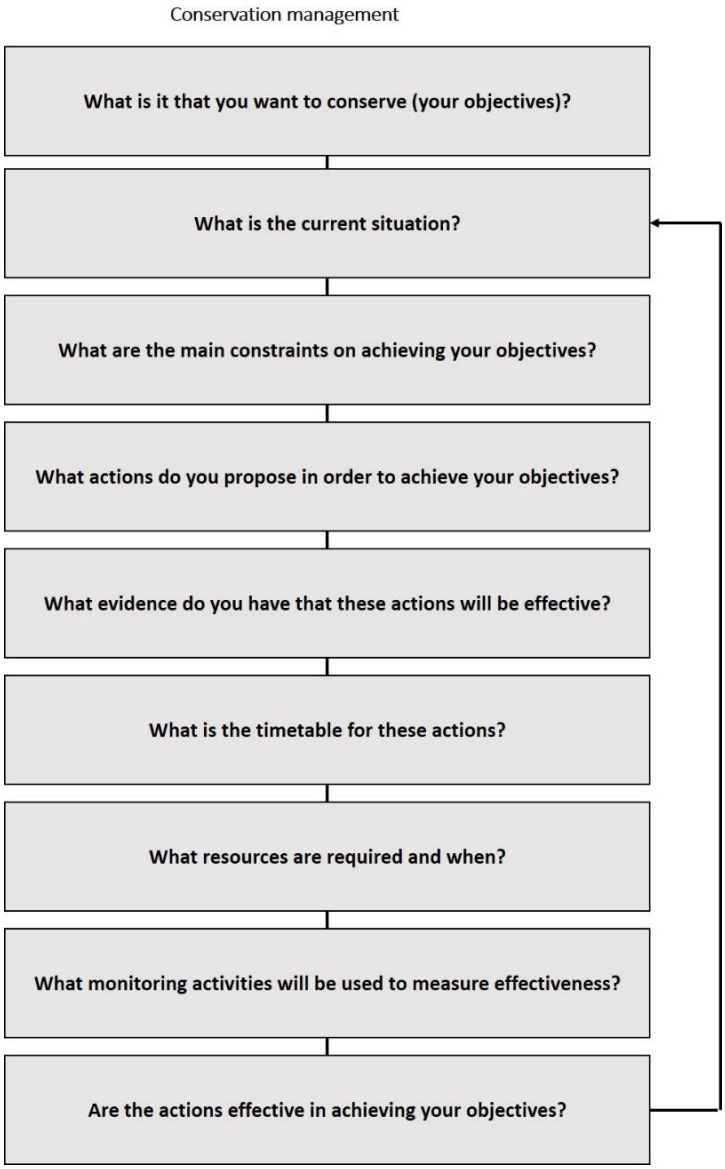



Figure 10: Flow diagram to assist in the production of a management action plan (adapted from Pullin, 2002).

## What's next? Future perspectives

This PhD thesis extends our knowledge about the ecology of cryptic species and how they are able to co-occur. Cryptic species of the *L. "marina"* complex seemed to be a lot more different than previously thought. Future research may be done on **other cryptic species of the *L. "marina"*** cryptic species complex or on **different species complexes** to see if coexistence is achieved in the same way and if differences can be found between the cryptic species.

Moreover, our knowledge about the studied cryptic species complex is still far from complete. Studying **life-history traits under more environmental conditions**, such as salinity fluctuations or broader temperature and salinity ranges etc., may give us more insights on niche differentiation between the species. Currently, we are starting a field survey to complete the information of Derycke et al. (2006) with information on smaller temporal scales and more geographical locations to elucidate the effect of abiotic conditions on the distribution of the cryptic species. We also want to incorporate the effect of **small-scale spatial differentiation** by looking at the receptacles and floating bladders of the algae. Moreover, biotic fluctuations, such as predation pressure, are ignored in our study and additional experiments or field work may be beneficial.

The outcome of the competition between the cryptic species is not that easily predictable and a lot of information is still lacking. Although we may never completely understand the details of these interactions, we may benefit from more research on **different combinations of species** (for instance all pairwise combinations within our studied species), which would undoubtedly allow a clearer picture of the competitively intransitive network between competing cryptic species of *L. marina*. An extensive experiment with different combinations of species at different abiotic conditions should give more insights about the intransitivity and the impact of abiotic changes.

In addition, the **exact nature of competition** is also still unclear for this cryptic species complex. Experimental studies can be conducted to test whether interference competition is important. For instance, when **mucus trails** of a nematode species are introduced to monospecific cultures of another species would affect that other species' population growth, this would indicate that interference competition may play a role in the interspecific interactions. Moreover, testing whether the cryptic species will change their niche when competition occurs, will also give us more insights in the mechanisms of competition. By

using Next Generation Sequencing to compare the **resource use of species** that occur alone and species **that suffer from competition**, we will be able to test if resource diversification will indeed be one of the important factors leading to coexistence. Also the relative importance of this resource differentiation in combination with dispersal can be tested in an experimental study in which resource diversity/composition as well as dispersal opportunities are varied in networks of interconnected patches (microcosms).

We only succeeded in revealing some aspects of the factors leading to coexistence of the cryptic species. More information on **intraspecific competition** will help to test the **neutral hypothesis**. If intraspecific competition is higher than, or equally high as interspecific competition, the ecological differences between the species may not be that important to their co-existence and neutral dynamics may play an important role. Detailed population studies, as well as studies on the effect of increasing intraspecific competition on resource use can be beneficial to understand this and can help to elucidate the importance of intraspecific versus interspecific competition.

More detailed studies on dispersal are extremely important as dispersal is a very important aspect to achieve coexistence and the link between passive and active dispersal is still unclear. An experimental setup in which the natural environment is imitated may help to understand this better. In mesocosms, we can create **different patches of algae on the sediment** and investigate the population dynamics of the cryptic species, with or without interspecific competition and with or without the presence of passive dispersal possibilities. We should also include **priority effects** here. These are important in the spatial storage paradigm and have already been shown to be important in *L. "marina"* (Derycke, et al., 2007). An experimental study on the competition between cryptic species when some species are already able to start a new population and others are just newly arriving, could clarify if priority effects are important in predicting the outcome of competition among *L. "marina"* species.

## General conclusion

Interspecific interactions frequently occur in the cryptic species complex of *L. "marina"* and a complex competitively intransitive network exists. Moreover, abiotic conditions affect the type and strength of these interactions. The cryptic species differ, however, in more aspects than previously thought and niche differentiation clearly occurs and may be one of the factors leading to coexistence of cryptic species. *L. "marina"* inhabits a temporally and spatially fluctuating environment in which dispersal may also play an important role. Differences in active dispersal abilities between the cryptic species combined with the differences in niche may indicate that the spatial storage effect is important to achieve coexistence within our cryptic species complex. We can't, however, exclude neutral dynamics and a combination of different paradigms to explain coexistence may be relevant. Understanding the ecology of the cryptic species may lead to deeper insights into important ecological processes and may help to activate more researchers to study this unknown world of cryptic species. Moreover, the ecological differences of the cryptic species lead to differences in ecosystem functioning, such as decomposition. As a result, it is extremely important to make people aware of cryptic biodiversity and its relevance for conservation biology.

# CITED LITERATURE

Abrams, B. I., Mitchell, M. J., 1980. Role of nematode-bacterial interactions in heterotrophic systems with emphasis on sewage sludge decomposition. Oikos 35(3): 404-410.

Abrams, P. A., 1986. Character displacement and niche shift analyzed using consumer-resource models of competition. Theoretical Population Biology 29(1): 107-160.

Agapow, P.-M., Bininda-Emonds, O. R., Crandall, K. A., Gittleman, J. L., Mace, G. M., Marshall, J. C., Purvis, A., 2004. The impact of species concept on biodiversity studies. The Quarterly Review of Biology 79(2): 161-179.

Aiken, C. M., Navarrete, S. A., 2014. Coexistence of competitors in marine metacommunities: environmental variability, edge effects, and the dispersal niche. Ecology 95(8): 2289-2302.

Akaike, H., 1981. Likelihood of a model and information criteria. Journal of Econometrics 16(1): 3-14.

Al-Naimi, F. A., Garrett, K. A., Bockus, W. W., 2005. Competition, facilitation, and niche differentiation in two foliar pathogens. Oecologia 143(3): 449-457.

Alford, R. A., Wilbur, H. M., 1985. Priority effects in experimental pond communities: competition between Bufo and Rana. Ecology 66(4): 1097-1105.

Alizon, S., Kucera, M., Jansen, V. A. A., 2008. Competition between cryptic species explains variations in rates of lineage evolution. Proceedings of the National Academy of Sciences of the US 105(34): 12382-12386.

Alkemade, R., Wielemaker, A., De Jong, S., Sandee, A. J. J., 1992. Experimental evidence for the role of bioturbation by the marine nematode *Diplolaimella dievengatensis* in stimulating the mineralization of Spartina anglica detritus. Marine Ecology Progress Series 90(2): 149-155.

Alkemade, R., Wielemaker, A., Hemminga, M. A., 1993. Correlation between nematode abundance and decomposition rate of *Spartina anglica* leaves. Marine Ecology Progress Series 99(3): 293-300.

Alves, A., Crous, P. W., Correia, A., Phillips, A. J. L., 2008. Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. Fungal Diversity 28(1): 1-13.

Amarasekare, P., Nisbet, R. M., 2001. Spatial heterogeneity, source-sink dynamics, and the local coexistence of competing species. The American Naturalist 158(6): 572-584.

Amarasekare, P., 2002. Interference competition and species coexistence. Proceedings of the Royal Society of London. Series B: Biological Sciences 269(1509): 2541-2550.

Amarasekare, P., 2003. Competitive coexistence in spatially structured environments: a synthesis. Ecology Letters 6(12): 1109-1122.

Amarasekare, P., 2004. Spatial dynamics of mutualistic interactions. Journal of Animal Ecology 73(1): 128-142.

Amarasekare, P., Hoopes, M. F., Mouquet, N., Holyoak, M., 2004. Mechanisms of coexistence in competitive metacommunities. The American Naturalist 164(3): 310-326.

Amato, A., Kooistra, W. H. C. F., Ghiron, J. H. L., Mann, D. G., Pröschold, T., Montresor, M., 2007. Reproductive isolation among sympatric cryptic species in marine diatoms. Protist 158(2): 193-207.

Anderson, M. J., 2001. A new method for non parametric multivariate analysis of variance. Austral Ecology 26(1): 32-46.

Anderson, M. J., 2004. PERMDISP: a FORTRAN computer program for permutatinoal analysis of multivariate dispersions (for any two-factor ANOVA design) using permutation tests. Department of Statistics, University of Auckland, New Zealand.

Andrassy, I., 1983. A taxonomic review of the suborder Rhaditina (Nematoda: Secernentea). Office de la Recherche Scientifique et Technique Outre-Mer, Paris

Anger, K., 1991. Effects of temperature and salinity on the larval development of the Chinese mitten crab *Eriocheir sinensis*(Decapoda: Grapsidae). Marine Ecology Progress Series 72(1): 103-110.

Appeltans, W., Ahyong, S. T., Anderson, G., Angel, M. V., Artois, T., Bailly, N., Bamber, R., Barber, A., Bartsch, I., Berta, A., et al., 2012. The magnitude of global marine species diversity. Current Biology 22(23): 2189-2202.

Armonies, W., 1988. Active emergence of meiofauna from intertidal sediment. Marine Ecology Progress Series 43(1): 151-159.

Atkinson, D., 1996. Ectotherm life-history responses to developmental temperature. In: Johnston, I.A., Bennet, A.F. (Eds.), Animals and temperature: Phenotypic and evolutionary adaptation. Cambridge University Press, Cambridge, pp. 183-204.

Bálint, M., Domisch, S., Engelhardt, C. H. M., Haase, P., Lehrian, S., Sauer, J., Theissinger, K., Pauls, S., Nowak, C., 2011. Cryptic biodiversity loss linked to global climate change. Nature Climate Change 1(6): 313-318.

Barata, C., Hontoria, F., Amat, F., Browne, R., 1996. Competition between sexual and parthenogenetic Artemia: Temperature and strain effects. Journal of Experimental Marine Biology and Ecology 196(1-2): 313-328.

Bastian, H. C., 1865. II. Monograph on the Anguillulidæ, or Free Nematoids, Marine, Land, and Freshwater; with Descriptions of 100 New Species. Transactions of the Linnean Society of London 25(2): 73-184.

Begon, M., 1996. Ecology: individuals, populations, and communities. 3rd ed. Blackwell Science Oxford.

Begon, M., Harper, J. L., Townsend, C. R., 1996. Interspecific competition. In: Harper., J., Townsend, C. (Eds.), Ecology: individuals, populations, and communities. Wiley-Blackwell, Tokyo, pp. 265 - 313.

Begon, M., Mortimer, M., Thompson, D. J., 2009. Population ecology: a unified study of animals and plants. Blackwell, Malden.

Beisner, B. E., Haydon, D. T., Cuddington, K., 2003. Alternative stable states in ecology. Frontiers in Ecology and the Environment 1(7): 376-382.

Bengtsson, J., 1987. Competitive dominance among Cladocera: Are single-factor explanations enough? Hydrobiologia 145(1): 245-257.

Benton, T. G., Grant, A., 2000. Evolutionary fitness in ecology: comparing measures of fitness in stochastic, density-dependent environments. Evolutionary Ecology Research 2: 769-789.

Bertness, M. D., Leonard, G. H., 1997. The role of positive interactions in communities: lessons from intertidal habitats. Ecology 78(7): 1976-1989.

Bhadury, P., Austen, M. C., Bilton, D. T., Lambshead, P. J. D., Rogers, A. D., Smerdon, G. R., 2008. Evaluation of combined morphological and molecular techniques for marine nematode (*Terschellingia spp.)* identification. Marine Biology 154(3): 509-518.

Bhishagratna, K. K., 1963. An English translation of the Sushruta Samhita based on the original Sanskrit text, with a full and comprehensive introduction, additional texts, different readings, notes, comparative views, index, glossary and plates, The Chowkhamba Sanskrit Studies. Chowkhamba Sanskrit Series Office, Varanasi.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K., Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22(3): 148-155.

Black II, R. W., Hairston, J. N. G., 1988. Predator driven changes in community structure. Oecologia 77(4): 468-479.

Blackburn, D. G., 1999. Viviparity and oviparity: evolution and reproductive strategies. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, New York, pp. 994-1003.

Blaustein, L., Margalit, J., 1996. Priority effects in temporary pools: nature and outcome of mosquito larva-toad tadpole interactions depend on order of entrance. Journal of Animal Ecology 65(1): 77-84.

Blouin, M. S., 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. International Journal for Parasitology 32(5): 527-531.

Bode, M., Bode, L., Armsworth, P. R., 2011. Different dispersal abilities allow reef fish to coexist. Proceedings of the National Academy of Sciences of the US 108(39): 16317-16321.

Boeckner, M., Sharma, J., Proctor, H., 2009. Revisiting the meiofauna paradox: dispersal and colonization of nematodes and other meiofaunal organisms in low- and high-energy environments. Hydrobiologia 624(1): 91-106.

Boileau, M. G., Hebert, P. D. N., Schwartz, S. S., 1992. Non-equilibrium gene frequency divergence: persistent founder effects in natural populations. Journal of Evolutionary Biology 5(1): 25-39.

Bolnick, D. I., Svanback, R., Araujo, M. S., Persson, L., 2007. Comparative support for the niche variation hypothesis that more generalized populations also are more heterogeneous. Proceedings of the National Academy of Sciences of the US 104(24): 10075-10079.

Bongers, T., 1990. The maturity index: an ecological measure of environmental disturbance based on nematode species composition. Oecologia 83(1): 14-19.

Bongers, T., Bongers, M., 1998. Functional diversity of nematodes. Applied Soil Ecology 10(3): 239-251.

Bonte, D., Van Dyck, H., Bullock, J. M., Coulon, A., Delgado, M., Gibbs, M., Lehouck, V., Matthysen, E., Mustin, K., Saastamoinen, M., 2011. Costs of dispersal. Biological Reviews 87(2): 290-312.

Bowen, B. W., Nelson, W. S., Avise, J. C., 1993. A molecular phylogeny for marine turtles: trait mapping, rate assessment, and conservation relevance. Proceedings of the National Academy of Sciences of the US 90(12): 5574-5577.

Bowler, D. E., Benton, T. G., 2005. Causes and consequences of animal dispersal strategies: relating individual behaviour to spatial dynamics. Biological Reviews 80(02): 205-225.

Brakefield, P. M., Kesbeke, F., 1997. Genotype–environment interactions for insect growth in constant and fluctuating temperature regimes. Proceedings of the Royal Society of London. Series B: Biological Sciences 264(1382): 717-723.

Breitburg, D. L., Baxter, J. W., Hatfield, C. A., Howarth, R. W., Jones, C. G., Lovett, G. M., Wigand, C., 1998. Understanding effects of multiple stressors: ideas and challenges. In: P., M.L., G., P.M. (Eds.), Successes, limitations, and frontiers in ecosystem science. Springer, New York, pp. 416-431.

Briggs, J. C., 1994. Species diversity: land and sea compared. Systematic Biology 43(1): 130-135.

Brown, W.L., Wilson, E.O., 1956. Character displacement. Systematic Zoology 5 (2): 49-64.

Bruno, J. F., Stachowicz, J. J., Bertness, M. D., 2003. Inclusion of facilitation into ecological theory. Trends in Ecology & Evolution 18(3): 119-125.

Bürger, R., Schneider, K. A., Willensdorfer, M., 2006. The conditions for speciation through intraspecific competition. Evolution 60(11): 2185-2206.

Burnell, A. M., Houthoofd, K., O'Hanlon, K., Vanfleteren, J.R., 2005. Alternate metabolism during the dauer stage of the nematode *Caenorhabditis elegans*. Experimental Gerontology 40: 850-856

Burrows, C. J., 1990. Processes of vegetation change. Springer Netherlands, Heidelberg.

Buys, B., 2014. Dispersal of marine nematodes: through air, water and sediment. Ghent University, Marine Biology: Biology Master Thesis.

Cabreiro, F., Gems, D., 2013. Worms need microbes too: microbiota, health and aging in Caenorhabditis elegans. EMBO Molecular Medicine 5(9): 1300-1310.

Cadotte, M. W., Mai, D. V., Jantz, S., Collins, M. D., Keele, M., Drake, J. A., 2006. On Testing the Competition-Colonization Trade-Off in a Multispecies Assemblage. The American Naturalist 168(5): 704-709.

Cadotte, M. W., 2007. Competition-colonization trade-offs and disturbance effects at multiple scales. Ecology 88(4): 823-829.

Calcagno, V., Mouquet, N., Jarne, P., David, P., 2006. Coexistence in a metacommunity: the competition–colonization trade-off is not dead. Ecology Letters 9(8): 897-907.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Tumbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5): 335-336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6(8): 1621-1624.

Capstick, C. K., 1959. The distribution of free-living nematodes in relation to salinity in the middle and upper reaches of the River Blyth estuary. Journal of Animal Ecology 28(2): 189-210.

Cardinale, B. J., Palmer, M. A., Collins, S. L., 2002. Species diversity enhances ecosystem functioning through interspecific facilitation. Nature 415(6870): 426-429.

Carman, K. R., Fry, B., 2002. Small-sample methods for delta C and delta N analysis of the diets of marsh meiofaunal species using natural-abundance and tracer-addition isotope techniques. Marine Ecology Progress Series 240: 85-92.

Case, T. J., Gilpin, M. E., 1974. Interference competition and niche theory. Proceedings of the National Academy of Sciences of the US 71(8): 3073.

Chandler, G. T., Fleeger, J. W., 1983. Meiofaunal colonization of azoic estuarine sediment in Louisiana: Mechanisms of dispersal. Journal of Experimental Marine Biology and Ecology 69(2): 175-188.

Chandler, G. T., Fleeger, J. W., 1987. Facilitative and inhibitory interactions among estuarine meiobenthic harpacticoid copepods. Ecology 68(6): 1906-1919.

Chang, C.-H., Lin, S.-M., Chen, J.-H., 2008. Molecular systematics and phylogeography of the gigantic earthworms of the *Metaphire formosae* species group (Clitellata, Megascolecidae). Molecular Phylogenetics and Evolution 49(3): 958-968.

Chen, J., Caswell-Chen, E. P., 2004. Facultative vivipary is a life-history trait in *Caenorhabditis elegans*. Journal of Nematology 36: 107-113.

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., Collman, R. G., Bushman, F. D., Li, H., 2012. Associating microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics 28(16): 2106-2113.

Chesson, P., 1991. A need for niches? Trends in Ecology & Evolution 6(1): 26-28.

Chesson, P., 2000a. General theory of competitive coexistence in spatially-varying environments. Theoretical Population Biology 58(3): 211-237.

Chesson, P., 2000b. Mechanisms of maintenance of species diversity. Annual Review of Ecology and Systematics 31: 343-366.

Chilton, N. B., Gasser, R. B., Beveridge, I., 1995. Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). International Journal for Parasitology 25(5): 647-651.

Chróst, R. J., 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, Microbial enzymes in aquatic environments. Springer, New York, pp. 29-59.

Ciros-Pérez, J., Carmona, M. J., Serra, M., 2001. Resource competition between sympatric sibling rotifer species. Limnology and Oceanography 46(6): 1511-1523.

Clarke, K. R., Gorley, R. N., 2006. PRIMER v6: User manual/tutoriaPrimer-E, Plymouth.

Clavero, M., García-Berthou, E., 2005. Invasive species are a leading cause of animal extinctions. Trends in Ecology and Evolution 20(3): 110-110.

Clobert, J., Le Galliard, J. F., Cote, J., Meylan, S., Massot, M., 2009. Informed dispersal, heterogeneity in animal dispersal syndromes and the dynamics of spatially structured populations. Ecology Letters 12(3): 197-209.

Cohen, D., Levin, S. A., 1991. Dispersal in patchy environments: the effects of temporal and spatial structure. Theoretical Population Biology 39(1): 63-99.

Colborn, J., Crabtree, R. E., Shaklee, J. B., Pfeiler, E., Bowen, B. W., 2001. The evolutionary enigma of bonefishes (*Albula spp*.): cryptic species and ancient separations in a globally distributed shorefish. Evolution 55(4): 807-820.

Colinet, H., Nguyen, T. T. A., Cloutier, C., Michaud, D., Hance, T., 2007. Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. Insect Biochemistry and Molecular Biology 37(11): 1177-1188.

Commito, J. A., Currier, C. A., Kane, L. R., Reinsel, K. A., Ulm, I. M., 1995. Dispersal dynamics of the bivalve Gemma gemma in a patchy environment. Ecological Monographs 65(7): 1-20.

Commito, J. A., Tita, G., 2002. Differential dispersal rates in an intertidal meiofauna assemblage. Journal of Experimental Marine Biology and Ecology 268(2): 237-256.

Coomans, A., 2000. Nematode systematics: past, present and future. Nematology 2(1): 3-7.

Coomans, A., 2002. Present status and future of nematode systematics. Nematology 4(5): 573-582.

Cossins, A. R., Bowler, K., 1987. Temperature biology of animals. Chapman and Hall, London.

Costanza, R., Fisher, B., Mulder, K., Liu, S., Christopher, T., 2007. Biodiversity and ecosystem services: A multi-scale empirical study of the relationship between species richness and net primary production. Ecological Economics 61(2): 478-491.

Cothran, R. D., Noyes, P., Relyea, R. A., 2015. An empirical test of stable species coexistence in an amphipod species complex. Oecologia 178(3): 1-13.

Coull, B. C., 1999. Role of meiofauna in estuarine soft bottom habitats. Australian Journal of Ecology 24(4): 327-343.

Coulson, T., Godfray, H. C. J., 2007. Single-species dynamics. In: May, R.M.C., McLean, A.R. (Eds.), Theoretical ecology: principles and applications. Oxford University Press, Oxford, pp. 17 - 34.

Covich, A. P., Palmer, M. A., Crowl, T. A., 1999. The role of benthic invertebrate species in freshwater ecosystems: zoobenthic species influence energy flows and nutrient cycling. BioScience 49(2): 119-127.

Covich, A. P., Austen, M. C., Bärlocher, F., Chauvet, E., Cardinale, B. J., Biles, C. L., Inchausti, P., Dangles, O., Solan, M., Gessner, M. O., 2004. The role of biodiversity in the functioning of freshwater and marine benthic ecosystems. BioScience 54(8): 767-775.

Cracraft, J., 1983. Species concepts and speciation analysis. In: Johnston, R.F. (Ed.), Current Ornithology. Springer US, New York, pp. 159-187.

Crombie, A. C., 1947. Interspecific competition. Journal of Animal Ecology 16(1): 44-73.

Czárán, T. L., Hoekstra, R. F., Pagie, L., 2002. Chemical warfare between microbes promotes biodiversity. Proceedings of the National Academy of Sciences of the US 99(2): 786-790.

Dailidiene, I., Davuliene, L., 2006. Spatial and temporal variation of water salinity in the Curonian Lagoon. Special Publication of the Hydrographic Society 55: 178 - 182.

Danovaro, R., Manini, E., Fabiano, M., 2002. Exoenzymatic activity and organic matter composition in sediments of the Northern Adriatic Sea: response to a river plume. Microbial Ecology 44(3): 235-251.

Danovaro, R., Gambi, C., Dell'Anno, A., Corinaldesi, C., Fraschetti, S., Vanreusel, A., Vincx, M., Gooday, A. J., 2008. Exponential decline of deep-sea ecosystem functioning linked to benthic biodiversity loss. Current Biology 18(1): 1-8.

Darwin, C., 1859. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London.

de Leon, G. P. P., Nadler, S. A., 2010. What we don't recognize can hurt us: a plea for awareness about cryptic species. Journal of Parasitology 96(2): 453-464.

De Ley, I. T., Mundo-Ocampo, M., Yoder, M., De Ley, P., 2007. Nematodes from vernal pools in the Santa Rosa Plateau Ecological Reserve, California I. *Hirschmanniella santarosae sp.*

*n*.(Nematoda: Pratylenchidae), a cryptic sibling species of *H. pomponiensis* Abdel-Rahman & Maggenti, 1987. Nematology 9(3): 405-429.

De Meester, L., Gomez, A., Okamura, B., Schwenk, K., 2002. The Monopolization Hypothesis and the dispersal-gene flow paradox in aquatic organisms. Acta Oecologica 23(3): 121-135.

De Meester, N., Derycke, S., Bonte, D., Moens, T., 2011. Salinity effects on the coexistence of cryptic species: A case study on marine nematodes. Marine Biology 158(12): 2717-2726.

De Meester, N., Derycke, S., Moens, T., 2012. Differences in time until dispersal between cryptic species of a marine nematode species complex. PLoS ONE 7(8): e42674.

De Meester, N., Derycke, S., Rigaux, A., Moens, T., 2015a. Temperature and salinity induce differential responses in life histories of cryptic nematode species. Journal of Experimental Marine Biology and Ecology 472: 54-62.

De Meester, N., Derycke, S., Rigaux, A., Moens, T., 2015b. Active dispersal is differentially affected by inter-and intraspecific competition in closely related nematode species. Oikos 124(5): 561-570.

De Meester, N., dos Santos, G. A. P., Rigaux, A., Valdes, Y., Derycke, S., Moens, T., 2015c. Daily Temperature Fluctuations Alter Interactions between Closely Related Species of Marine Nematodes PloS One 10(7): e0131625.

De Meester, N., Gingold, R., Rigaux, A., Derycke, S. & Moens, T., Cryptic diversity and ecosystem functioning: a complex tale of differential effects on decomposition, accepted in Oecologia.

De Mesel, I., Derycke, S., Swings, J., Vincx, M., Moens, T., 2003. Influence of bacterivorous nematodes on the decomposition of cordgrass. Journal of Experimental Marine Biology and Ecology 296(2): 227-242.

De Mesel, I., Derycke, S., Moens, T., Van der Gucht, K., Vincx, M., Swings, J., 2004. Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. Environmental Microbiology 6(7): 733-744.

De Mesel, I., Derycke, S., Swings, J., Vincx, M., Moens, T., 2006. Role of nematodes in decomposition processes: Does within-trophic group diversity matter? Marine Ecology Progress Series 321: 157 - 166.

De Queiroz, K., 2007. Species concepts and species delimitation. Systematic Biology 56(§): 879-886

Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M., Moens, T., 2005. Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. Marine Ecology Progress Series 300: 91-103.

Derycke, S., Backeljau, T., Vlaeminck, C., Vierstraete, A., Vanfleteren, J., Vincx, M., Moens, T., 2006. Seasonal dynamics of population genetic structure in cryptic taxa of the *Pellioditis marina* complex (Nematoda: Rhabditida). Genetica 128(1): 307-321.

Derycke, S., Backeljau, T., Vlaeminck, C., Vierstraete, A., Vanfleteren, J., Vincx, M., Moens, T., 2007a. Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, Monhysteridae) reveals high levels of molecular diversity. Marine Biology 151(5): 1799-1812.

Derycke, S., Van Vynckt, R., Vanoverbeke, J., Vincx, M., Moens, T., 2007b. Colonization patterns of Nematoda on decomposing algae in the estuarine environment: Community assembly and genetic structure of the dominant species *Pellioditis marina*. Limnology and Oceanography 52(3): 992-1001.

Derycke, S., Fonseca, G., Vierstraete, A., Vanfleteren, J., Vincx, M., Moens, T., 2008a. Disentangling taxonomy within the *Rhabditis (Pellioditis) marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. Zoological Journal of the Linnean Society 152(1): 1-15.

Derycke, S., Remerie, T., Backeljau, T., Vierstraete, A., Vanfleteren, J., Vincx, M., 2008b. Phylogeography of the *Rhabditis (Pellioditis) marina* species complex: evidence for long-distance dispersal, and for range expansions and restricted gene flow in the northeast Atlantic. Molecular Ecology 17(14): 3306-3322.

Derycke, S., De Ley, P., Tandingan De Ley, I., Holovachov, O., Rigaux, A., Moens, T., 2010. Linking DNA sequences to morphology: cryptic diversity and population genetic structure in the marine nematode *Thoracostoma trachygaster* (Nematoda, Leptosomatidae). Zoologica Scripta 39(3): 276-289.

Derycke, S., Sheibani Tezerji, R., Rigaux, A., Moens, T., 2012. Investigating the ecology and evolution of cryptic marine nematode species through quantitative real time PCR of the ribosomal ITS region. Molecular Ecology Resources 12: 607 - 619.

Derycke, S., Backeljau, T., Moens, T., 2013. Dispersal and gene flow in free-living marine nematodes. Frontiers in Zoology 10(1): 1.

Derycke, S., De Meester, N., Rigaux, A., Creer, S., Bik, H., Thomas, W. K., Moens, T., 2016. Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability. Molecular Ecology: doi: 10.1111/mec.13597.

Descamps-Julien, B., Gonzalez, A., 2005. Stable coexistence in a fluctuating environment: an experimental demonstration. Ecology 86(10): 2815-2824.

Diaz, H., Bevilacqua, M., 1986. Larval development of *Aratus pisonii* (Milne Edwards)(Brachyura, Grapsidae) from marine and estuarine environments reared under different salinity conditions. Journal of Coastal Research 2(1): 43-49.

Díaz, S., Fargione, J., Chapin, F. S., Tilman, D., 2006. Biodiversity loss threatens human well-being. PloS Biology 4(8): 1300-1305.

Dietrich, G., Kalle, K., 1957. Allgemeine Meereskunde: Eine Einführung in die Ozeanographie. 8 ed. Gebrüder Bornträger, Berlin.

Doncaster, C. P., Clobert, J., Doligez, B., Danchin, E., Gustafsson, L., 1997. Balanced dispersal between spatially varying local populations: an alternative to the source-sink model. The American Naturalist 150(4): 425-445.

dos Santos, G. A. P., Derycke, S., Fons ca-Genevois, V. G., Coelho, L., Correia, M. T. S., Moens, T., 2008. Differential effects of food availability on population growth and fitness of three species of estuarine, bacterial-feeding nematodes. Journal of Experimental Marine Biology and Ecology 355(1): 27-40.

dos Santos, G. A. P., Derycke, S., Genevois, V. G. F., Coelho, L. C. B. B., Correia, M. T. S., Moens, T., 2009. Interactions among bacterial-feeding nematode species at different levels of food availability. Marine Biology 156(4): 629-640.

Dunson, W. A., Travis, J., 1991. The role of abiotic factors in community organization. American Naturalist 138(5): 1067-1091.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., Mearns, L. O., 2000. Climate extremes: observations, modeling, and impacts. Science 289(5487): 2068-2074.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16): 2194-2200.

Egler, F. E., 1954. Vegetation science concepts I. Initial floristic composition, a factor in old-field vegetation development with 2 figs. Plant Ecology 4(6): 412-417.

Ehrlich, P. R., Ehrlich, A. H., 1981. Extinction: the causes and consequences of the disappearance of species. Random House, New York.

Ellstrand, N. C., Schierenbeck, K. A., 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? Proceedings of the National Academy of Sciences of the US 97(13): 7043-7050.

Elmer, K. R., Bonett, R. M., Wake, D. B., Lougheed, S. C., 2013. Early Miocene origin and cryptic diversification of South American salamanders. BMC Evolutionary Biology 13(1): 1.

Estifanos, T. K., Traunspurger, W., Peters, L., 2013. Selective feeding in nematodes: a stable isotope analysis of bacteria and algae as food sources for free-living nematodes. Nematology 15: 1-13.

Ettema, C. H., 1998. Soil nematode diversity: species coexistence and ecosystem function. Journal of Nematology 30(2): 159-169.

Feldberg, K., Groth, H., Wilson, R., Schäfer-Verwimp, A., Heinrichs, J., 2004. Cryptic speciation in Herbertus (Herbertaceae, Jungermanniopsida): range and morphology of *Herbertus sendtneri* inferred from nrITS sequences. Plant Systematics and Evolution 249(3-4): 247-261.

Felix, M.-A., Braendle, C., Cutter, A. D., 2014. A streamlined system for species diagnosis in *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. PloS One 9(4): e94723.

Ferris, H., Venette, R. C., Van Der Meulen, H. R., Lau, S. S., 1998. Nitrogen mineralization by bacterial-feeding nematodes: verification and measurement. Plant and Soil 203(2): 159-171.

Findlay, S., Tenore, K., 1982. Nitrogen source for a detritivore: detritus substrate versus associated microbes. Science 218(4570): 371-373.

Finlay, B. J., Maberly, S. C., Cooper, J. I., 1997. Microbial diversity and ecosystem function. Oikos 80(2): 209-213.

Fischer, K., Kölzow, N., Höltje, H., Karl, I., 2011. Assay conditions in laboratory experiments: is the use of constant rather than fluctuating temperatures justified when investigating temperature-induced plasticity? Oecologia 166(1): 23 - 33.

Fišer, Ž., Altermatt, F., Zakšek, V., Knapič, T., Fišer, C., 2015. Morphologically Cryptic Amphipod Species Are "Ecological Clones" at Regional but Not at Local Scale: A Case Study of Four Niphargus Species. PloS One 10(7): e0134384.

Fonderie, P., Steel, H., Moens, T., Bert, W., 2013. Experimental induction of intraspecific morphometric variability in a single population of *Halicephalobus cf. gingivalis* may surpass total interspecific variability. Nematology 15(5): 529-544.

Fonseca, D. M., Hart, D. D., 1996. Density-dependent dispersal of black fly neonates is mediated by flow. Oikos 75(1): 49-58.

Fonseca, G., Derycke, S., Moens, T., 2008. Integrative taxonomy in two free-living nematode species complexes. Biological Journal of the Linnean Society 94(4): 737-753.

Fonseca, V. G., Carvalho, G. R., Sung, W., Johnson, H. F., Power, D. M., Neill, S. P., Packer, M., Blaxter, M. L., Lambshead, P. J. D., Thomas, W. K., Creer, S., 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. Nature Communications 1.

Foran, J. A., 1986. A comparison of the life history features of a temperate and a subtropical *Daphnia* species. Oikos 46(2): 185-193.

Fouquet, A., Vences, M., Salducci, M. D., Meyer, A., Marty, C., Blanc, M., Gilles, A., 2007. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. Molecular Phylogenetics and Evolution 43(2): 567-582.

Fournier, F., Boivin, G., 2000. Comparative dispersal of *Trichogramma evanescens* and *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) in relation to environmental conditions. Environmental Entomology 29(1): 55-63.

Freckman, D. W., 1988. Bacterivorous nematodes and organic-matter decomposition. Agriculture, Ecosystems & Environment 24(1): 195-217.

Fujii, K., 1968. Studies on interspecies competition between the azuki bean weevil and the southern cowpea weevil. III. Some characteristics of strains of two species. Researches on Population Ecology 10(1): 87-98.

Funk, W. C., Caminer, M., Ron, S. R., 2011. High levels of cryptic species diversity uncovered in Amazonian frogs. Proceedings of the Royal Society of London B: Biological Sciences 282(1816): doi:10.1098/rspb.2011.1653.

Futuyma, D. J., 2005. Evolution. Sinauer Associates, Sunderland.

Gabaldón, C., Carmona, M. J., Montero-Pau, J., Serra, M., 2015. Long-term competitive dynamics of two cryptic rotifer species: diapause and fluctuating conditions. PloS One 10(4): e0124406.

Gallucci, F., Moens, T., Vanreusel, A., Fonseca, G., 2008. Active colonisation of disturbed sediments by deep-sea nematodes: evidence for the patch mosaic model. Marine Ecology Progress Series 367: 173-183.

Gandon, S., 1999. Kin competition, the cost of inbreeding and the evolution of dispersal. Journal of Theoretical Biology 200(4): 345-364.

Gandon, S., Rousset, F., 1999. Evolution of stepping-stone dispersal rates. Proceedings of the Royal Society of London B: Biological Sciences 266(1437): 2507-2513.

Garcia, E. A., Mittelbach, G. G., 2008. Regional coexistence and local dominance in Chaoborus: species sorting along a predation gradient. Ecology 89(6): 1703-1713.

Gaspar, J. M., Thomas, W. K., 2015. FlowClus: efficiently filtering and denoising pyrosequenced amplicons. BMC Bioinformatics 16(1): 1.

Gaudy, R., Guerin, J. P., Moraitou-Apostolopoulou, M., 1982. Effect of temperature and salinity on the population dynamics of *Tisbe holothuriae* Humes (Copepoda: Harpacticoida) fed on two different diets. Journal of Experimental Marine Biology and Ecology 57(2): 257-271.

Geller, J. B., 1999. Decline of a native mussel masked by sibling species invasion. Conservation Biology 13(3): 661-664.

Gerhardt, H. C., 2005. Acoustic spectral preferences in two cryptic species of grey treefrogs: implications for mate choice and sensory mechanisms. Animal Behaviour 70(1): 39-48.

Gienapp, P., Teplitsky, C., Alho, J. S., Mills, J. A., Merilä, J., 2008. Climate change and evolution: disentangling environmental and genetic responses. Molecular Ecology 17(1): 167-178.

Gilbert, J. A., Jansson, J. K., Knight, R., 2014. The Earth Microbiome project: successes and aspirations. BMC Biology 12(1): 69.

Gilchrist, G. W., 1995. Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. American Naturalist 146(2): 252-270.

Gilpin, M. E., 1974. Intraspecific competition between *Drosophila* larvae in serial transfer systems. Ecology 55(5): 1154-1159.

Gingold, R., Ibarra-Obando, S. E., Rocha-Olivares, A., 2011. Spatial aggregation patterns of free-living marine nematodes in contrasting sandy beach micro-habitats. Journal of the Marine Biological Association of the United Kingdom 91(3): 615-622.

Glasby, C. J., Wei, N. W. V., Gibb, K. S., 2013. Cryptic species of Nereididae (Annelida : Polychaeta) on Australian coral reefs. Invertebrate Systematics 27(3): 245-264.

Gómez, A., Carmona, M. J., Serra, M., 1997. Ecological factors affecting gene flow in the *Brachionus plicatilis* complex (Rotifera). Oecologia 111(3): 350-356.

Gómez, A., Serra, M., Carvalho, G. R., Lunt, D. H., 2002. Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). Evolution 56(7): 1431-1444.

Gómez, Á., Temprano, M., Serra, M., 1995. Ecological genetics of a cyclical parthenogen in temporary habitats. Journal of Evolutionary Biology 8(5): 601-622.

Goulson, D., Darvill, B., 2004. Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? Apidologie 35(1): 55-63.

Grainger, J. N. R., 1958. First stages in the adaptation of poikilotherms to temperature change. In: Prosser, C. (Ed.), Physiological adaptation American Physiological Society, Washington, DC, pp. 79–91.

Granger, H., 1985. The scala naturae and the continuity of kinds. Phronesis 30(2): 181-200.

Grosemans, T., Morris, K., Thomas, W. K., Rigaux, A., Moens, T., Derycke, S., 2016. Mitogenomics reveals high synteny and long evolutionary histories of sympatric cryptic nematode species. Ecology and Evolution Early view: 10.1002/ece3.1975.

Guilini, K., Soltwedel, T., van Oevelen, D., Vanreusel, A., 2011. Deep-Sea Nematodes Actively Colonise Sediments, Irrespective of the Presence of a Pulse of Organic Matter: Results from an In-Situ Experiment. PloS One 6(4): e18912.

Hanski, I., Ranta, E., 1983. Coexistence in a patchy environment: three species of *Daphnia* in rock pools. Journal of Animal Ecology 52(1): 263-279.

Hanski, I., 1999. Metapopulation ecology. Oxford University Press, Oxford.

Harper, J. L., 1961. Approaches to the study of plant competition, Symposia of Society for Experimental Biology, pp. 1-39.

Harrison, G. W., 1979. Stability under environmental stress: resistance, resilience, persistence, and variability. American Naturalist 113(5): 659-669.

Harvey, S. C., 2009. Non dauer larval dispersal in *Caenorhabditis elegans*. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312(3): 224-230.

Hassell, M. P., Comins, H. N., May, R. M., 1994. Species coexistence and self-organizing spatial dynamics. Nature 370: 290-292.

Hastings, A., 1980. Disturbance, coexistence, history, and competition for space. Theoretical Population Biology 18(3): 363-373.

Hebert, P. D. N., Crease, T. J., 1980. Clonal coexistence in *Daphnia pulex* (Leydig): another planktonic paradox. Science 207: 1363-1365.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the US 101(41): 14812-14817.

Heip, C. H. R., Vincx, M., Vranken, G., 1985. The ecology of marine nematodes. Oceanography and Marine Biology: an Annual Review 23: 399 - 489.

Henry, C. S., 1994. Singing and cryptic speciation insects. Trends in Ecology & Evolution 9(10): 388-392.

Henry, C. S., Wells, M. L. M., Simon, C. M., 1999. Convergent evolution of courtship songs among cryptic species of the *Carnea* group of green lacewings (Neuroptera: Chrysopidae: Chrysoperla). Evolution 53(4): 1165-1179.

Hey, J., Waples, R. S., Arnold, M. L., Butlin, R. K., Harrison, R. G., 2003. Understanding and confronting species uncertainty in biology and conservation. Trends in Ecology & Evolution 18(11): 597-603.

Heynssens, C., 2015. Experimental research on pheromones in sympatrically occurring cryptic species. Ghent University, Marine Biology: Biology Master Thesis

Higashi, M., Patten, B. C., 1989. Dominance of indirect causality in ecosystems. The American Naturalist 133(2): 288-302.

Hoberg, E. P., Monsen, K. J., Kutz, S., Blouin, M. S., 1999. Structure, biodiversity, and historical biogeography of nematode faunas in holarctic ruminants: morphological and molecular diagnoses for *Teladorsagia boreoarcticus n. sp.(*Nematoda: Ostertagiinae), a dimorphic cryptic species in muskoxen (Ovibos moschatus). The Journal of Parasitology 85(5): 910-934.

Höckelmann, C., Moens, T., Jüttner, F., 2004. Odor compounds from cyanobacterial biofilms acting as attractants and repellents for free-living nematodes. Limnology and Oceanography 49(5): 1809-1819.

Højgaard, D. P., 1998. Impact of temperature, salinity and light on hatching of eggs of *Anisakis simplex* (Nematoda, Anisakidae), isolated by a new method, and some remarks on survival of larvae. Sarsia: a Norwegian Journal of Marine Biology 83: 21 - 28.

Hopper, B. E., Fell, J. W., Cefalu, R. C., 1973. Effect of temperature on life cycles of nematodes associated with the mangrove (Rhizophora mangle) detrital system. Marine Biology 23(4): 293-296.

Hubas, C., Sachidhanandam, C., Rybarczyk, H., Lubarsky, H. V., Rigaux, A., Moens, T., Paterson, D. M., 2010. Bacterivorous nematodes stimulate microbial growth and exopolymer production in marine sediment microcosms. Marine Ecology Progress Series 419: 85-94.

Hubbell, S. P., 2001. The unified neutral theory of biodiversity and biogeography. Princeton University Press, New Jersey.

Hubbell, S. P., 2005. Neutral theory in community ecology and the hypothesis of functional equivalence. Functional Ecology 19(1): 166-172.

Hubbell, S. P., 2006. Neutral theory and the evolution of ecological equivalence. Ecology 87(6): 1387-1398.

Huettel, R. N., 1986. Chemical communicators in nematodes. Journal of Nematology 18(1): 3-8.

Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N., Vellend, M., 2008. Ecological consequences of genetic diversity. Ecology Letters 11(6): 609-623.

Hughes, C. L., Hill, J. K., Dytham, C., 2003. Evolutionary trade-offs between reproduction and dispersal in populations at expanding range boundaries. Proceedings of the Royal Society of London. Series B: Biological Sciences 270(Suppl 2): S147.

Hugo, L., 2012. Wie is competitief het sterkst? Een competitiestudie met 4 cryptische soorten bij verschillende omgevingsfactoren. Ghent University, Marine Biology: Biology Bachelor Thesis.

Huisman, J., Weissing, F. J., 1999. Biodiversity of plankton by species oscillations and chaos. Nature 402(6760): 407-410.

Hutchinson, G. E., MacArthur, R. H., 1959. A theoretical ecological model of size distributions among species of animals. American Naturalist 93(869): 117-125.

Huxel, G. R., 1999. Rapid displacement of native species by invasive species: effects of hybridization. Biological Conservation 89(2): 143-152.

Ilieva-Makulec, K., 2001. A comparative study of the life strategies of two bacterial-feeding nematodes under laboratory conditions. III. Influence of the initial nematode density on the interactions of *Arobeloides nanus*(de Man 1880) Anderson and *Dolichorhabditis dolichura*(Schneider 1866) Andrassy 1983 in mixed cultures. Polish Journal of Ecology 49(2): 137-144.

Ims, R. A., Hjermann, D. O., 2001. Condition-dependent dispersal. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (Eds.), Dispersal. Oxford University Press, Oxford.

Inglis, W. G., Coles, J. W., 1961. The species of *Rhabditis* (Nematoda) found in rotting seaweed on British beaches. Bulletin of the British Museum (Natural History) 7(6): 320-333.

Jensen, P., 1981. Phyto-chemical sensitivity and swimming behavior of the free-living marine nematode *Chromadorita tenuis*. Marine Ecology Progress Series(2).

Jensen, T. C., Hessen, D. O., Faafeng, B. A., 2001. Biotic and abiotic preferences of the cladoceran invader *Limnosida frontosa*. Hydrobiologia 442(1): 89-99.

Johnson, L. L., Shick, J. M., 1977. Effects of fluctuating temperature and immersion on asexual reproduction in the intertidal sea anemone *Hauplanella luciae* (Verrill) in laboratory culture. Journal of Experimental Marine Biology and Ecology 28(2): 141-149.

Jolliffe, P. A., 2000. The replacement series. Journal of Ecology 88(3): 371-385.

Jones, G., 1997. Acoustic signals and speciation: the roles of natural and sexual selection in the evolution of cryptic species. Advances in the Study of Behaviour 26: 317-354.

Jones, G., Holderied, M. W., 2007. Bat echolocation calls: adaptation and convergent evolution. Proceedings of the Royal Society of London B: Biological Sciences 274(1612): 905-912.

Jonsson, M., Malmqvist, B., 2003. Mechanisms behind positive diversity effects on ecosystem functioning: testing the facilitation and interference hypotheses. Oecologia 134(4): 554-559.

Kaiser, M., Attrill, M. J., Jennings, S., Thomas, D. N., Barnes, D. K. A., Brierley, A. S., Polunin, N. V. C., Raffaelli, D. G., Williams, P., 2005. Marine Ecology: processes, systems, and impacts. Oxford University Press Oxford.

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Mayer, W. E., Sommer, R. J., 2012. Description of three *Pristionchus species* (Nematoda: Diplogastridae) from Japan that form a cryptic species complex with the model organism P. pacificus. Zoological science 29(6): 403-417.

Kiontke, K. C., Fitch, D. H. A., 2010. Phenotypic Plasticity: Different Teeth for Different Feasts. Current Biology 20(17): R710 - R712.

Kiontke, K. C., Félix, M.-A., Ailion, M., Rockman, M. V., Braendle, C., Pénigault, J.-B., Fitch, D. H., 2011. A phylogeny and molecular barcodes for *Caenorhabditis*, with numerous new species from rotting fruits. BMC Evolutionary Biology 11(1): 339.

Klautau, M., Russo, C. A. M., Lazoski, C., Boury-Esnault, N., Thorpe, J. P., Solé-Cava, A. M., 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula.* Evolution 53(5): 1414-1422.

Knowlton, N., 1993. Sibling species in the sea. Annual Review of Ecology and Systematics 24(1): 189-216.

Kronfeld-Schor, N., Dayan, T., 1999. The dietary basis for temporal partitioning: food habits of coexisting *Acomys* species. Oecologia 121(1): 123-128.

Kuussaari, M., Nieminen, M., Hanski, I., 1996. An experimental study of migration in the Glanville fritillary butterfly *Melitaea cinxia*. Journal of Animal Ecology 65(6): 791-801.

Lachnit, T., Meske, D., Wahl, M., Harder, T., Schmitz, R., 2011. Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. Environmental Microbiology 13(3): 655-665.

Laird, R. A., Schamp, B. S., 2006. Competitive intransitivity promotes species coexistence. The American Naturalist 168(2): 182-193.

Laird, R. A., Schamp, B. S., 2008. Does local competition increase the coexistence of species in intransitive networks. Ecology 89(1): 237-247.

Lale, N. E. S., Vidal, S., 2001. Intraspecific and interspecific competition in *Callosobruchus maculatus* (F.) and *Callosobruchus subinnotatus* (Pic) on stored bambara groundnut, *Vigna subterranea* (L.) Verdcourt. Journal of Stored Products Research 37(4): 329-338.

Lambin, X., Aars, J., Piertney, S. B., 2001. Dispersal, intraspecific competition, kin competition and kin facilitation: a review of the empirical evidence. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (Eds.), Dispersal. Oxford University Press, Oxford, pp. 110-122.

Lambshead, P. J. D., Boucher, G., 2003. Marine nematode deep sea biodiversity–hyperdiverse or hype? Journal of Biogeography 30(4): 475-485.

Lambshead, P. J. D., 2004. Marine nematode diversity. In: Chen, Z.X., Chen, W.Y., Chen, S.Y., Dickson, S.W. (Eds.), Nematology: Advances and Perspectives Tsinghua University Press Beijing, pp. 436–467.

Landry, C., Geyer, L., Arakaki, Y., Uehara, T., Palumbi, S. R., 2003. Recent speciation in the Indo–West Pacific: rapid evolution of gamete recognition and sperm morphology in cryptic species of sea urchin. Proceedings of the Royal Society of London. Series B: Biological Sciences 270(1526): 1839-1847.

Langellotto, G. A., Denno, R. F., Ott, J. R., 2000. A trade-off between flight capability and reproduction in males of a wing-dimorphic insect. Ecology 81(3): 865-875.

Langenheder, S., Székely, A. J., 2011. Species sorting and neutral processes are both important during the initial assembly of bacterial communities. The ISME Journal 5(7): 1086-1094.

Lawler, S. P., Morin, P. J., 1993. Temporal overlap, competition, and priority effects in larval anurans. Ecology 74(1): 174-182.

Lawton, J. H., Brown, V. K., 1994. Redundancy in ecosystems. In: Schulze, E.-D., Mooney, H.A. (Eds.), Biodiversity and Ecosystem Function. Springer-Verlag, Berlin Heidelberg.

Leavitt, S. D., Fernández-Mendoza, F., Pérez-Ortega, S., Sohrabi, M., Divakar, P. K., Lumbsch, H. T., St Clair, L. L., 2013. DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7: 1-22.

Lee, J. J., Tietjen, J. H., Mastropaolo, C., Rubin, H., 1977. Food quality and the heterogeneous spatial distribution of meiofauna. Helgoland Marine Research 30(1): 272-282.

Lefébure, T., Douady, C. J., Gouy, M., Trontelj, P., Briolay, J., Gibert, J., 2006. Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. Molecular Ecology 15(7): 1797-1806.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., Holt, R. D., Shurin, J. B., Law, R., Tilman, D., 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecology Letters 7(7): 601-613.

Leibold, M. A., McPeek, M. A., 2006. Coexistence of the niche and neutral perspectives in community ecology. Ecology 87(6): 1399-1410.

Leibold, M. A., 2008. Ecology: return of the niche. Nature 454(7200): 39-41.

Lenaerts, I., Walker, G., Van Hoorebeke, L., Gems, D., Vanfleteren, J., 2008. Dietary restriction of *Caenorhabditis elegans* by axenic culture reflects nutritional requirement for constituents

provided by metabolically active microbes. Journals of Gerontology Series A Biological Sciences and Medical Sciences 63: 242-252.

Levine, J. M., Rees, M., 2002. Coexistence and relative abundance in annual plant assemblages: the roles of competition and colonization. The American Naturalist 160(4): 452-467.

Lieberman, B. S., Dudgeon, S., 1996. An evaluation of stabilizing selection as a mechanism for stasis. Palaeogeography, Palaeoclimatology, Palaeoecology 127(1): 229-238.

Light, D. M., Birch, M. C., Paine, T. D., 1983. Laboratory study of intraspecific and interspecific competition within and between two sympatric bark beetle species, *Ips pini* and *I. paraconfusus*. Zeitschrift für angewandte Entomologie 96(1-5): 233-241.

Lindenmayer, D., Hunter, M., 2010. Some guiding concepts for conservation biology. Conservation Biology 24(6): 1459-1468.

Lins, L., Vanreusel, A., Van Campenhout, J., Ingels, J., 2013. Selective settlement of deep-sea canyon nematodes after resuspension—an experimental approach. Journal of Experimental Marine Biology and Ecology 441: 110-116.

Litchman, E., Klausmeier, C. A., 2001. Competition of phytoplankton under fluctuating light. The American Naturalist 157(2): 170-187.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 ΔΔCT Method. Methods 25(4): 402-408.

Lomnicki, A., 1988. Individual variation in resource partitioning and population dynamics, Population ecology of individuals. Princeton University Press, New Jersey, pp. 20 - 42.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J. P., Hector, A., Hooper, D. U., Huston, M. A., Raffaelli, D., Schmid, B., 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294(5543): 804-808.

Loreau, M., 2004. Does functional redundancy exist? Oikos 104(3): 606-611.

Lowe, C., Kemp, S., Diaz-Avalos, C., Montagnes, D., 2006. How does salinity tolerance influence the distributions of *Brachionus plicatilis* sibling species? Marine Biology 150(3): 377-386.

Lurz, P. W. W., Garson, P. J., Wauters, L. A., 1997. Effects of temporal and spatial variation in habitat quality on red squirrel dispersal behaviour. Animal Behaviour 54(2): 427-435.

MacArthur, R., Levins, R., 1967. The limiting similarity, convergence, and divergence of coexisting species. American Naturalist 101: 377-385.

MacArthur, R. H., Wilson, E. O., 1967. The theory of island biogeography. Princeton University Press, New Jersey.

Magner, L. N., 2002. A history of the life sciences, revised and expanded. CRC Press, Boca Raton.

Malakhov, U. V., 1986. Nematodes: Structure, development, classification and phylogeny. Smithsonian Institution Scholary Press, Washington, 320 pp.

Mamilov, A. S., Byzov, B. A., Pokarzhevskii, A. D., Zvyagintsev, D. G., 2000. Regulation of the biomass and activity of soil microorganisms by microfauna. Microbiology 69(5): 612-621.

Marcott, S. A., Shakun, J. D., Clark, P. U., Mix, A. C., 2013. A reconstruction of regional and global temperature for the past 11,300 years. Science 339(6124): 1198-1201.

Mare, M. F., 1942. A study of a marine benthic community with special reference to the micro-organisms. Journal of the Marine Biological Association of the United Kingdom. 25(3): 517-554.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17(1).

Martinez, J. G., dos Santos, G. A. P., Derycke, S., Moens, T., 2012. Effects of cadmium on the fitness of, and interactions between, two bacterivorous nematode species. Applied Soil Ecology 56: 10-18.

Maslen, N. R., Convey, P., 2006. Nematode diversity and distribution in the southern maritime Antarctic—clues to history? Soil Biology and Biochemistry 38(10): 3141-3151.

Matz, C., Kjelleberg, S., 2005. Off the hook–how bacteria survive protozoan grazing. Trends in Microbiology 13(7): 302-307.

Mayden, R. L., 1997. A hierarchy of species concepts: the denouement in the saga of the species problem. In: Claridge, M.F., Dawah, H.A., Wilson, M.R. (Eds.), Species: The units of diversity. Chapman and Hall, London, pp. 381-423.

Mayer, F., Von Helversen, O., 2001. Sympatric distribution of two cryptic bat species across Europe. Biological Journal of the Linnean Society 74(3): 365-374.

Mayr, E., 1942. Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press, London.

Mayr, E., 1982. The growth of biological thought: diversity, evolution, and inheritanceHarvard University Press, London.

McPeek, M. A., Holt, R. D., 1992. The evolution of dispersal in spatially and temporally varying environments. American Naturalist 140(6): 1010-1027.

McPeek, M. A., Brown, J. M., 2000. Building a regional species pool: diversification of the Enallagma damselflies in eastern North America. Ecology 81(4): 904-920.

Meegaskumbura, M., Bossuyt, F., Pethiyagoda, R., Manamendra-Arachchi, K., Bahir, M., Milinkovitch, M. C., Schneider, C. J., 2002. Sri Lanka: an amphibian hot spot. Science 298(5592): 379-379.

Mevenkamp, L., Van Campenhout, J., Vanreusel, A., 2016. Experimental evidence for selective settlement of meiofauna from two distinct environments after sediment suspension. Journal of Experimental Marine Biology and Ecology 474: 195-203.

Meyer-Reil, L.-A., 1987. Seasonal and spatial distribution of extracellular enzymatic activities and microbial incorporation of dissolved organic substrates in marine sediments. Applied and Environmental Microbiology 53(8): 1748-1755.

Micoletzky, H., 1922. Freilebende Nematoden aus den treibenden Tangen der Sargassosee. Mitteilungen des Hamburger Zoologischen Museums und Institutes 39: 1-11.

Millennium Ecosystem Assessment, 2005. Ecosystems and human well-being: Synthesis. Island Press, Washington, DC.

Mills, L. S., Soulé, M. E., Doak, D. F., 1993. The keystone-species concept in ecology and conservation. BioScience 43(4): 219-224.

Moens, T., Vierstraete, A., Vanhove, S., Verbeke, M., Vincx, M., 1996. A handy method for measuring meiobenthic respiration. Journal of Experimental Marine Biology and Ecology 197(2): 177-190.

Moens, T., Vincx, M., 1997. Observations on the feeding ecology of estuarine nematodes. Journal of the Marine Biological Association of the United Kingdom 77(01): 211-227.

Moens, T., Vincx, M., 1998. On the cultivation of free-living marine and estuarine nematodes. Helgoland Marine Research 52(2): 115-139.

Moens, T., Verbeeck, L., De Mayer, A., Swings, J., Vincx, M., 1999. Selective attraction of marine bacterivorous nematodes to their bacterial food. Marine Ecology Progress Series 176: 165-178.

Moens, T., Herman, P. M. J., Verbeeck, L., Steyaert, M., Vincx, M., 2000. Predation rates and prey selectivity in two predacious estuarine nematodes. Marine Ecology Progress Series 205: 185-193.

Moens, T., Vincx, M., 2000a. Temperature, salinity and food thresholds in two brackish-water bacterivorous nematode species: assessing niches from food absorption and respiration experiments. Journal of Experimental Marine Biology and Ecology 243(1): 137-154.

Moens, T., Vincx, M., 2000b. Temperature and salinity constraints on the life cycle of two brackish-water nematode species. Journal of Experimental Marine Biology and Ecology 243(1): 115-135.

Moens, T., Yeates, G., De Ley, P., 2004. Use of carbon and energy sources by nematodes, Proceedings of the Fourth International Congress of Nematology, 8-13 June 2002, Tenerife, Spain, pp. 529-545.

Moens, T., dos Santos, G. A. P., Thompson, F., Swings, J., Fonsêca-Genevois, V., Vincx, M., De Mesel, I., 2005. Do nematode mucus secretions affect bacterial growth? Aquatic Microbial Ecology 40(1): 77-83.

Molbo, D., Machado, C. A., Sevenster, J. G., Keller, L., Herre, E. A., 2003. Cryptic species of fig-pollinating wasps: Implications for the evolution of the fig–wasp mutualism, sex allocation, and precision of adaptation. Proceedings of the National Academy of Sciences of the US 100(10): 5867-5872.

Mole, S., Zera, A. J., 1993. Differential allocation of resources underlies the dispersal-reproduction trade-off in the wing-dimorphic cricket, Gryllus rubens. Oecologia 93(1): 121-127.

Montero-Pau, J., Ramos-Rodríguez, E., Serra, M., Gómez, A., 2011. Long-term coexistence of rotifer cryptic species. PloS One 6(6): e21530.

Moritz, C., 1994. Defining 'evolutionarily significant units' for conservation. Trends in Ecology & Evolution 9(10): 373-375.

Mougi, A., Nishimura, K., 2005. Coexistence of competitive species with a stage-structured life cycle. Ecological Research 20(5): 581-589.

Mouquet, N., Loreau, M., 2002. Coexistence in metacommunities: the regional similarity hypothesis. The American Naturalist 159(4): 420-426.

Nascimento, F. J. A., Naslund, J., Elmgren, R., 2012. Meiofauna enhances organic matter mineralization in soft sediment ecosystems. Limnology and Oceanography 57(1): 338-346.

Neher, D. A., 2001. Role of nematodes in soil health and their use as indicators. Journal of Nematology 33(4): 161-168.

Neira, C., Sellanes, J., Levin, L. A., Arntz, W. E., 2001. Meiofaunal distributions on the Peru margin: relationship to oxygen and organic matter availability. Deep Sea Research Part I: Oceanographic Research Papers 48(11): 2453-2472.

Nicholson, A. J., 1954. An outline of the dynamics of animal populations. Australian Journal of Zoology 2(1): 9-65.

Norling, K., Rosenberg, R., Hulth, S., Grémare, A., Bonsdorff, E., 2007. Importance of functional biodiversity and species-specific traits of benthic fauna for ecosystem functions in marine sediment. Marine Ecology Progress Series 332: 11-23.

Obendorf, D. L., Beveridge, I., Andrews, R. H., 1991. Cryptic species in populations of Globocephaloides trifidospicularis Kung (Nematoda: Trichostrongyloidea), parasitic in macropodid marsupials. Transactions of the Royal Society of South Australia 115: 213-216.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., Suggests, M., 2007. The vegan package. Community ecology package 10.

Ólafsson, E., Moore, C. G., 1990. Control of meiobenthic abundance by macroepifauna in a subtidal muddy habitat. Marine Ecology Progress Series 65: 241-249.

Oro, D., Cam, E., Pradel, R., Martínez-Abraín, A., 2004. Influence of food availability on demography and local population dynamics in a long-lived seabird. Proceedings of the Royal Society Biological Sciences Series B 271(1537): 387-396.

Ortells, R., Gómez, A., Serra, M., 2003. Coexistence of cryptic rotifer species: ecological and genetic characterisation of *Brachionus plicatilis*. Freshwater Biology 48(12): 2194-2202.

Palmer, M. A., Gust, G., 1985. Dispersal of meiofauna in a turbulent tidal creek. Journal of Marine Research 43(1): 179-210.

Palmer, M. A., 1988. Dispersal of marine meiofauna: A review and conceptual model explaining passive transport and active emergence with implications for recruitment. Marine Ecology Progress Series 48(1): 81-91.

Palmer, M. A., Allan, J. D., Butman, C. A., 1996. Dispersal as a regional process affecting the local dynamics of marine and stream benthic invertebrates. Trends in Ecology & Evolution 11(8): 322-326.

Palumbi, S. R., Metz, E. C., 1991. Strong reproductive isolation between closely related tropical sea urchins (genus *Echinometra*). Molecular Biology and Evolution 8(2): 227-239.

Pan, W., 2001. Akaike's information criterion in generalized estimating equations. Biometrics 57(1): 120-125.

Parsons, P. A., 1994. Morphological stasis: an energetic and ecological perspective incorporating stress. Journal of Theoretical Biology 171(4): 409-414.

Peng, Y.-Q., Duan, Z.-B., Yang, D.-R., Rasplus, J.-Y., 2008. Co-occurrence of two Eupristina species on *Ficus altissima* in Xishuangbanna, SW China. Symbiosis (Rehovot) 45(1): 9-14.

Perez-Portela, R., Almada, V., Turon, X., 2013. Cryptic speciation and genetic structure of widely distributed brittle stars (Ophiuroidea) in Europe. Zoologica Scripta 42(2): 151-169.

Perrin, N., Goudet, J., 2001. Inbreeding, kinship, and the evolution of natal dispersal. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (Eds.), Dispersal. Oxford University Press, Oxford, pp. 123-142.

Pétavy, G., David, J. R., Gibert, P., Moreteau, B., 2001. Viability and rate of development at different temperatures in *Drosophila*: a comparison of constant and alternating thermal regimes. Journal of Thermal Biology 26(1): 29-39.

Pfenninger, M., Schwenk, K., 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evolutionary Biology 7(1): 121.

Pickett, S. T., 1980? Non-equilibrium coexistence of plants. Bulletin of the Torrey Botanical Club 107 (2): 238-248.

Pinto, J. D., Oatman, E. R., Platner, G. R., 1986. *Trichogramma pretiosum* and a new cryptic species occurring sympatrically in Southwestern North America (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America 79(6): 1019-1028.

Postma-Blaauw, M. B., De Vries, F. T., De Goede, R. G. M., Bloem, J., Faber, J. H., Brussaard, L., 2005. Within-trophic group interactions of bacterivorous nematode species and their effects on the bacterial community and nitrogen mineralization. Oecologia 142(3): 428-439.

Powers, T., 2004. Nematode molecular diagnostics: from bands to barcodes. Annual Review of Phytopathology 42: 367-383.

Prada, C., McIlroy, S. E., Beltrán, D. M., Valint, D. J., Ford, S. A., Hellberg, M. E., Coffroth, M. A., 2014. Cryptic diversity hides host and habitat specialization in a gorgonian-algal symbiosis. Molecular Ecology 23(13): 3330-3340.

Pulliam, H. R., 1988. Sources, sinks, and population regulation. American Naturalist 132(5): 652-661.

Pullin, A. S., 2002. Conservation Biology. Cambridge University Press, Cambridge.

R Development Core Team, 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Ralin, D. B., 1968. Ecological and reproductive differentiation in the cryptic species of the Hyla versicolor complex (Hylidae). Southwestern Naturalist 13(3): 283-299.

Ravaoarimanana, I. B., Tiedemann, R., Montagnon, D., Rumpler, Y., 2004. Molecular and cytogenetic evidence for cryptic speciation within a rare endemic Malagasy lemur, the Northern Sportive Lemur (*Lepilemur septentrionalis*). Molecular Phylogenetics and Evolution 31(2): 440-448.

Razgour, O., Clare, E. L., Zeale, M. R., Hanmer, J., Schnell, I. B., Rasmussen, M., Gilbert, T. P., Jones, G., 2011. High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. Ecology and Evolution 1(4): 556-570.

Resh, V. H., Unzicker, J. D., 1975. Water quality monitoring and aquatic organisms: the importance of species identification. Journal of Water Pollution Control Federation 47(1): 9-19.

Riemann, F., Schrage, M., 1978. The mucus-trap hypothesis on feeding of aquatic nematodes and implications for biodegradation and sediment texture. Oecologia 34(1): 75-88.

Rissler, L. J., Apodaca, J. J., 2007. Adding more ecology into species delimitation: ecological niche models and phylogeography help define cryptic species in the black salamander (Aneides flavipunctatus). Systematic Biology 56(6): 924-942.

Ristau, K., Steinfartz, S., Traunspurger, W., 2013. First evidence of cryptic species diversity and significant population structure in a widespread freshwater nematode morphospecies (Tobrilus gracilis). Molecular Ecology 22(17): 4562-4575.

Rocha-Olivares, A., Fleeger, J. W., Foltz, D. W., 2001. Decoupling of molecular and morphological evolution in deep lineages of a meiobenthic harpacticoid copepod. Molecular Biology and Evolution 18(6): 1088-1102.

Rocha-Olivares, A., Fleeger, J. W., Foltz, D. W., 2004. Differential tolerance among cryptic species: A potential cause of pollutant-related reductions in genetic diversity. Environmental Toxicology and Chemistry 23(9): 2132-2137.

Rohland, N., Reich, D., Mallick, S., Meyer, M., Green, R. E., Georgiadis, N. J., Roca, A. L., Hofreiter, M., 2010. Genomic DNA sequences from mastodon and woolly mammoth reveal deep speciation of forest and savanna elephants. PloS Biology 8(12): 2916.

Rojas-Echenique, J., Allesina, S., 2011. Interaction rules affect species coexistence in intransitive networks. Ecology 92(5): 1174-1180.

Rojas, M., 1992. The species problem and conservation: what are we protecting? Conservation Biology 6(2): 170-178.

Ronce, O., 2007. How does it feel to be like a rolling stone? Ten questions about dispersal evolution. Annual Review of Ecology Evolution and Systematics 38: 231-253.

Saastamoinen, M., Hanski, I., 2008. Genotypic and environmental effects on flight activity and oviposition in the Glanville fritillary butterfly. American Naturalist 171(6): 701-712.

Sáez, A. G., Probert, I., Geisen, M., Quinn, P., Young, J. R., Medlin, L. K., 2003. Pseudo-cryptic speciation in coccolithophores. Proceedings of the National Academy of Sciences 100(12): 7163-7168.

Sáez, A. G., Lozano, E., 2005. Body doubles - cryptic species: as we discover more examples of species that are morphologically indistinguishable, we need to ask why and how they coexist. Nature 433(7022): 111.

Savage, V. M., Gillooly, J. F., Brown, J. H., West, G. B., Charnov, E. L., 2004. Effects of body size and temperature on population growth. The American Naturalist 163(3): 429-441.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., 2012. Fiji: an open-source platform for biological-image analysis. Nature methods 9(7): 676-682.

Schluter, D., 2009. Evidence for ecological speciation and its alternative. Science 323 (5915): 737-741.

Schoener, T. W., 1974. Resource partitioning in ecological communities. Science 185(4145): 27-39.

Schratzberger, M., Whomersley, P., Warr, K., Bolam, S. G., Rees, H. L., 2004. Colonisation of various types of sediment by estuarine nematodes via lateral infaunal migration: a laboratory study. Marine Biology 145(1): 69-78.

Schröder, T., Walsh, E. J., 2007. Cryptic speciation in the cosmopolitan *Epiphanes senta* complex (Monogononta, Rotifera) with the description of new species. Hydrobiologia 593(1): 129-140.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol 12(6): R60.

Service, S. K., Bell, S. S., 1987. Density-influenced active dispersal of harpacticoid copepods. Journal of Experimental Marine Biology and Ecology 114(1): 49-62.

Seymour, M. K., Wright, K., Doncaster, C., 1983. The action of the anterior feeding apparatus of *Caenorhabditis elegans* (Nematoda: Rhabditida). Journal of zoology 201(4): 527-539.

Shulman, M. J., Ogden, J. C., Ebersole, J. P., McFarland, W. N., Miller, S. L., Wolf, N. G., 1983. Priority effects in the recruitment of juvenile coral reef fishes. Ecology 64(6): 1508-1513.

Siepielski, A. M., Hung, K.-L., Bein, E. E., McPeek, M. A., 2010. Experimental evidence for neutral community dynamics governing an insect assemblage. Ecology 91(3): 847-857.

Singer, C., 1931. Short History of Biology. Oxford University Press, London.

Sison-Mangus, M. P., Jiang, S., Tran, K. N., Kudela, R. M., 2014. Host-specific adaptation governs the interaction of the marine diatom, *Pseudo-nitzschia* and their microbiota. The ISME Journal 8(1): 63-76.

Šlapeta, J., López-García, P., Moreira, D., 2006. Global dispersal and ancient cryptic species in the smallest marine eukaryotes. Molecular Biology and Evolution 23(1): 23-29.

Snyder, R. E., Chesson, P., 2003. Local dispersal can facilitate coexistence in the presence of permanent spatial heterogeneity. Ecology Letters 6(4): 301-309.

Solan, M., Cardinale, B. J., Downing, A. L., Engelhardt, K. A. M., Ruesink, J. L., Srivastava, D. S., 2004. Extinction and ecosystem function in the marine benthos. Science 306(5699): 1177-1180.

Solé-Cava, A., Klautau, M., Boury-Esnault, N., Borojecic, R., Thorpe, J., 1991. Genetic evidence for cryptic speciation in allopatric populations of two cosmopolitan species of the calcareous sponge genus *Clathrina*. Marine Biology 111(3): 381-386.

Soliveres, S., DeSoto, L., Maestre, F. T., Olano, J. M., 2010. Spatio-temporal heterogeneity in abiotic factors modulate multiple ontogenetic shifts between competition and facilitation. Perspectives in Plant Ecology, Evolution and Systematics 12(3): 227-234.

Soulé, M. E., 1985. What is conservation biology? A new synthetic discipline addresses the dynamics and problems of perturbed species, communities, and ecosystems. BioScience 35(11): 727-734.

Southwood, T., 1977. Habitat, the templet for ecological strategies? Journal of Animal Ecology 46(2): 337-365.

Srinivasan, J., von Reuss, S. H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M. C., O'Doherty, O. G., Edison, A. S., Sternberg, P. W., Schroeder, F. C., 2012. A Modular Library of Small Molecule Signals Regulates Social Behaviors in *Caenorhabditis elegans*. PloS Biology 10(1): e1001237.

Steyaert, M., Garner, N., van Gansbeke, D., Vincx, M., 1999. Nematode communities from the North Sea: environmental controls on species diversity and vertical distribution within the sediment. Journal of the Marine Biological Association of the United Kingdom 79(2): 253-264.

Stuart, B. L., Inger, R. F., Voris, H. K., 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biology Letters 2(3): 470-474.

Sudhaus, W., 1974. Nematoden (insbesondere Rhabditiden) des Strandanwurfs und ihre Beziehungen zu Krebsen. Faunistisch Okologische Mitteilungen 4: 365-400.

Sudhaus, W., Kiontke, K., 2007. Comparison of the cryptic nematode species *Caenorhabditis brenneri sp. n.* and *C. remanei* (Nematoda: Rhabditidae) with the stem species pattern of the Caenorhabditis Elegans group. Zootaxa 1456: 45-62.

Sudhaus, W., 2011. Phylogenetic systematisation and catalogue of paraphyletic "Rhabditidae" (Secernentea, Nematoda). Journal of Nematode Morphology and Systematics 14: 113-178.

Suits, D. B., 1957. Use of dummy variables in regression equations. Journal of the American Statistical Association 52(280): 548-551.

Svanbäck, R., Bolnick, D. I., 2007. Intraspecific competition drives increased resource use diversity within a natural population. Proceedings of the Royal Society B: Biological Sciences 274(1611): 10.1098/rspb.2006.0198

Thiel, M., Gutow, L., 2005. The ecology of rafting in the marine environment. II. The rafting organisms and community. Oceanography and Marine Biology: An Annual Review 43: 279-418.

Thomas, C. F. G., Jepson, P. C., 1999. Differential aerial dispersal of linyphiid spiders from a grass and a cereal field. Journal of Arachnology 27(1): 294-300.

Thomas, M. C., Lana, P. C., 2011. A new look into the small-scale dispersal of free-living marine nematodes. Zoologia (Curitiba) 28(4): 449-456.

Tietjen, J. H., Lee, J. J., Rullman, J., Greengart, A., Trompeter, J., 1970. Gnotobiotic culture and physiological ecology of the marine nematode Rhabditis marina Bastian. Limnology and Oceanography 15(4): 535-543.

Tietjen, J. H., Lee, J. J., 1972. Life cycles of marine nematodes. Oecologia 10(2): 167-176.

Tietjen, J. H., Lee, J. J., 1975. Axenic culture and uptake of dissolved organic substances by the marine nematode, *Rhabditis marina* Bastian. Cahiers de Biologie Marine XVI: 685 - 693.

Tilman, D., 1976. Ecological competition between algae: experimental confirmation of resource-based competition theory. Science 192(4238): 463-465.

Tilman, D., 1977. Resource competition between plankton algae: an experimental and theoretical approach. Ecology 58(2): 338-348.

Travis, J. M. J., Murrell, D. J., Dytham, C., 1999. The evolution of density–dependent dispersal. Proceedings of the Royal Society of London. Series B: Biological Sciences 266(1431): 1837-1842.

Trewick, S. A., 1998. Sympatric cryptic species in New Zealand Onychophora. Biological Journal of the Linnean Society 63(3): 307-329.

Ullberg, J., Olafsson, E., 2003. Effects of biological disturbance by *Monoporeia affinis* (Amphipoda) on small-scale migration of marine nematodes in low-energy soft sediments. Marine Biology 143(5): 867-874.

Ullberg, J., Ólafsson, E., 2003. Free-living marine nematodes actively choose habitat when descending from the water column. Marine Ecology Progress Series 260: 141-149.

Ullberg, J., 2004. Dispersal in free-living, marine, benthic nematodes: passive or active processes? Stockholm University, Department of Zoology: PhD Thesis.

Urban-Malinga, B., Gheskiere, T., Degraer, S., Derycke, S., Opalinski, K., Moens, T., 2008. Gradients in biodiversity and macroalgal wrack decomposition rate across a macrotidal, ultradissipative sandy beach. Marine Biology 155(1): 79-90.

Vafeiadou, A.-M., Materatski, P., Adão, H., De Troch, M., Moens, T., 2014. Resource utilization and trophic position of nematodes and harpacticoid copepods in and adjacent to *Zostera noltii* beds. Biogeosciences 11(14): 4001-4014.

Valiente-Banuet, A., Verdú, M., 2008. Temporal shifts from facilitation to competition occur between closely related taxa. Journal of Ecology 96(3): 489-494.

Van Butsel, J., 2014. Alternative methods for the assesment of pollution in marine nematodes: behavioral analysis versus developmental and reproductive assays. Ghent University, Marine Biology: Biology Master Thesis.

Van Campenhout, J., Derycke, S., Moens, T., Vanreusel, A., 2014. Differences in life-histories refute ecological equivalence of cryptic species and provide clues to the origin of Bathyal *Halomonhystera* (Nematoda). PloS One 9(11): e111889.

Van Daele, T., 2014. Dispersal in cryptic species of the *Litoditis marina* (Nematoda) species complex: are settlement and resuspension purely passive processes. Ghent University, Marine Biology: Biology Master Thesis.

Van Damme, L., 2015. Velddistributie van cryptische soorten: kunnen nauw gerelateerde soorten samen voorkomen in de natuur? Ghent University, Marine Biology: Biology Bachelor Thesis.

van de Voorde, T. F. J., van der Putten, W. H., Martijn Bezemer, T., 2011. Intra- and interspecific plant–soil interactions, soil legacies and priority effects during old-field succession. Journal of Ecology 99(4): 945-953.

Van Gaever, S., Moodley, L., Beer, D. d., Vanreusel, A., 2006. Meiobenthos at the Arctic Håkon Mosby Mud Volcano with a parental caring nematode thriving in sulphide-rich sediments. Marine Ecology Progress Series 321: 143-155.

Van Tienderen, P. H., 1991. Evolution of generalists and specialist in spatially heterogeneous environments. Evolution 45(6): 1317-1331.

Van Valen, L., 1971. Group selection and the evolution of dispersal. Evolution 25(4): 591-598.

Vanaverbeke, J., Gheskiere, T., Vincx, M., 2000. The meiobenthos of subtidal sandbanks on the Belgian Continental Shelf (Southern Bight of the North Sea). Estuarine Coastal and Shelf Science 51(5): 637-649.

Vanelslander, B., Creach, V., Vanormelingen, P., Ernst, A., Chepurnov, V. A., Sahan, E., Muyzer, G., Stal, L. J., Vyverman, W., Sabbe, K., 2009. Ecological differentiation between sympatric pseudocryptic species in the estuarine benthic diatom *Navicula phyllepta* (Bacillariophyceae). Journal of Phycology 45(6): 1278-1289.

Vanfleteren, J. R., 1980. Nematodes as nutritional models. In: Zuckerman, B. (Ed.), Nematodes as biological models. Academic Press, New York, pp. 47-80.

Vanschoenwinkel, B., De Vries, C., Seaman, M., Brendonck, L., 2007. The role of metacommunity processes in shaping invertebrate rock pool communities along a dispersal gradient. Oikos 116(8): 1255-1266.

Veltjen, E., 2012. Experimenteel onderzoek naar de hybridisatiemogelijkheden tussen cryptische soorten. Ghent University, Marine Biology: Biology Bachelor Thesis.

Violle, C., Nemergut, D. R., Pu, Z., Jiang, L., 2011. Phylogenetic limiting similarity and competitive exclusion. Ecology Letters 14(8): 728 - 787.

Vlaams-Nederlandse Scheldecommissie, Werkgroep Onderzoek en Monitoring Scheldemonitor. http://www.scheldemonitor.be/dataportal/.

Vogler, A. P., Desalle, R., 1994. Diagnosing units of conservation management. Conservation Biology 8(2): 354-363.

Vranken, G., Heip, C., 1983. Calculation of the intrinsic rate of natural increase, rm, with *Rhabditis marina* Bastian 1865 (Nematoda). Nematologica 29: 468– 477.

Vranken, G., Herman, P. M. J., Heip, C., 1988. Studies of the life-history and energetics of marine and brackish-water nematodes. Oecologia 77(3): 296-301.

Vrijenhoek, R. C., Schutz, S. J., Gustafson, R. G., Lutz, R. A., 1994. Cryptic species of deep-sea clams (Mollusca: Bivalvia: Vesicomyidae) from hydrothermal vent and cold-water seep environments. Deep Sea Research Part I: Oceanographic Research Papers 41(8): 1171-1189.

Walker, B. H., 1992a. Biodiversity and ecological redundancy. Conservation Biology 6(1): 18-23.

Walker, M. A., 1992b. Redundancy in collaborative dialogue. Proceedings of the 14th conference on Computational linguistics 1: 345-351.

Walters, K., Bell, S. S., 1986. Diel patterns of active vertical migration in seagrass meiofauna. Marine Ecology-Progress Series 34: 95-103.

Walther, G.-R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J. C., Fromentin, J.-M., Hoegh-Guldberg, O., Bairlein, F., 2002. Ecological responses to recent climate change. Nature 416(6879): 389-395.

Wang, K.-H., McSorley, R., Marshall, A. J., Gallaher, R. N., 2004. Nematode community changes associated with decomposition of *Crotalaria juncea* amendment in litterbags. Applied Soil Ecology 27(1): 31-45.

Warner, R. R., Chesson, P. L., 1985. Coexistence mediated by recruitment fluctuations: a field guide to the storage effect. American Naturalist 125(6): 769-787.

Waser, P. M., 1985. Does competition drive dispersal? Ecology 66(4): 1170-1175.

Wear, R. G., Haslett, S. J., Alexander, N. L., 1986. Effects of temperature and salinity on the biology of *Artemia fransiscana* Kellogg from Lake Grassmere, New Zealand. 2. Maturation, fecundity, and generation times. Journal of Experimental Marine Biology and Ecology 98(1): 167-183.

Webb, C. O., Ackerly, D. D., McPeek, M. A., Donoghue, M. J., 2002. Phylogenies and community ecology. Annual Review of Ecology and Systematics 33: 475-505.

Webb, C. O., Gilbert, G. S., Donoghue, M. J., 2006. Phylodiversity-dependent seedling mortality, size structure, and disease in a Bornean rain forest. Ecology 87(sp7): 123-131.

Wellborn, G., Cothran, R., 2007. Niche diversity in crustacean cryptic species: complementarity in spatial distribution and predation risk. Oecologia 154(1): 175-183.

Westerbom, M., Kilpi, M., Mustonen, O., 2002. Blue mussels, *Mytilus edulis*, at the edge of the range: population structure, growth and biomass along a salinity gradient in the north-eastern Baltic Sea. Marine Biology 140(5): 991-999.

Wetzel, M., Weber, A., Giere, O., 2002. Re-colonization of anoxic/sulfidic sediments by marine nematodes after experimental removal of macroalgal cover. Marine Biology 141(4): 679-689.

Wiens, J. A., 2001. The landscape context of dispersal. In: Clobert, J., Danchin, E., Dhondt, A.A., Nichols, J.D. (Eds.), Dispersal Oxford University Press, Oxford, pp. 96-109.

Wieser, W., 1953. Die Beziehung zwischen Mundhöhlengestalt, Ernährungsweise und Vorkommen bei freilebenden marinen Nematoden. Arkiv för Zoologi 4: 439-484.

Wilke, T., Pfenninger, M., 2002. Separating historic events from recurrent processes in cryptic species: phylogeography of mud snails (*Hydrobia spp*.). Molecular Ecology 11(8): 1439-1451.

Wilkins, J., 2006. Species, kinds, and evolution. Reports of the National Center for Science Education 26(4): 36-45.

Williams, H. C., Ormerod, S. J., Bruford, M. W., 2006. Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae). Molecular Phylogenetics and Evolution 40(2): 370-382.

Williams, W. D., 1998. Salinity as a determinant of the structure of biological communities in salt lakes. Hydrobiologia 381(1): 191-201.

Willig, M. R., Kaufman, D. M., Stevens, R. D., 2003. Latitudinal gradients of biodiversity: pattern, process, scale, and synthesis. Annual Review of Ecology, Evolution, and Systematics 34: 273-309.

Winegardner, A. K., Jones, B. K., Ng, I. S. Y., Siqueira, T., Cottenie, K., 2012. The terminology of metacommunity ecology. Trends in Ecology and Evolution 27: 253-254.

Winston, M. R., 1995. Co-occurrence of morphologically similar species of stream fishes. American Naturalist 145(4): 527-545.

Witt, J. D., Hebert, P. D., 2000. Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. Canadian Journal of Fisheries and Aquatic Sciences 57(4): 687-698.

Wolters, V., 2001. Biodiversity of soil animals and its function. European Journal of Soil Biology 37(4): 221-227.

Worm, B., Barbier, E. B., Beaumont, N., Duffy, J. E., Folke, C., Halpern, B. S., Jackson, J. B. C., Lotze, H. K., Micheli, F., Palumbi, S. R., 2006. Impacts of biodiversity loss on ocean ecosystem services. Science 314(5800): 787-790.

Yachi, S., Loreau, M., 1999. Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. Proceedings of the National Academy of Sciences of the US 96(4): 1463-1468.

Yassin, A., Capy, P., Madi-Ravazzi, L., Ogereau, D., David, J. R., 2008. DNA barcode discovers two cryptic species and two geographical radiations in the invasive drosophilid *Zaprionus indianus*. Molecular Ecology Resources 8(3): 491-501.

Yeates, G. W., Coleman, D. C., 1982. Nematodes in decomposition. In: Freckman, D.W. (Ed.), Nematodes in soil ecosystems. Univeristy of Texas Press, Austin, pp. 55-80.

Yeates, G. W., Bongers, T., 1999. Nematode diversity in agroecosystems. Agriculture, Ecosystems & Environment 74(1): 113-135.

Yoder, M., De Ley, I. T., King, I. W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L., De Ley, P., 2006. DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. Nematology 8(3): 367-376.

Zera, A. J., Harshman, L. G., 2001. The physiology of life history trade-offs in animals. Annual Review of Ecology and Systematics 32: 95-126.

Zhang, D. Y., Lin, K., Hanski, I., 2004. Coexistence of cryptic species. Ecology Letters 7(3): 165 - 169.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5): 614-620.

Zhou, H., 2001. Effects of leaf litter addition on meiofaunal colonization of azoic sediments in a subtropical mangrove in Hong Kong. Journal of Experimental Marine Biology and Ecology 256(1): 99-121.

Zoetendal, E. G., Akkermans, A. D. L., De Vos, W. M., 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Applied and Environmental Microbiology 64(10): 3854-3859.

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., Smith, G. M., 2009. Mixed effects models and extensions in ecology with R. Springer, New York.

# PUBLICATION LIST

## A1 – peer reviewed articles

**De Meester, N**. & Bonte, D. (2010) Information use and density-dependent dispersal in an agrobiont spider. Behavioral Ecology 21(5): 992-998.

Bonte, D., **De Meester, N.** & Matthysen E. (2011) Selective integration advantages when transience is costly: immigration behaviour in an agrobiont spider Animal Behaviour 81(4): 837 – 841.

**De Meester, N.**, Derycke, S., Bonte, D. & Moens, T. (2011) Salinity effects on the coexistence of cryptic species: A case study on marine nematodes. Marine Biology 158: 2717-2726.

**De Meester, N., Derycke, S. & Moens, T.** (2012) Differences in time until dispersal between cryptic species of a marine nematode species complex. PloS One, 7, e42674.

Macheriotou, L.; Braeckman, B.P.; Rigaux, A.; **De Meester, N**.; Moens, T. & Derycke, S. (2015). Intraspecific functional trait variability does not result in higher fitness under thermal stress in a free-living marine nematode. Journal of Experimental Marine. Biology and. Ecology 472: 14-23.

**De Meester, N**., Derycke, S., Rigaux, A. & Moens, T. (2015) Active dispersal is differentially affected by inter- and intraspecific competition in closely related nematode species. Oikos 124: 561-570.

**De Meester, N.,** Derycke, S., Rigaux, A. & Moens, T. (2015) Temperature and salinity induce differential responses in life histories of cryptic nematode species. Journal of Experimental Marine Biology and Ecology 472: 54-62.

**De Meester, N**., dos Santos, G.A.P., Rigaux, A., Valdes, Y., Derycke, S. & Moens, T. (2015) Daily temperature fluctuations alter interactions between closely related species of marine nematodes, PLoS One 10: e0131625.

Martinez, J.G., Paran, G.P.,· Rizon, R., **De Meester, N**., Moens, T. (2016) Copper effects on soil nematodes and their possible impact on leaf litter decomposition: A microcosm approach. European Journal of Soil Biology 73:1-7.

Derycke, S., **De Meester, N.,** Rigaux, A., Creer, S., Bik, H., Thomas, W.K. & Moens, T. (2016) Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability, Molecular Ecology: doi: 10.1111/mec.13597

**De Meester, N.,** Gingold, R., Rigaux, A., Derycke, S. & Moens, T. (accepted) Cryptic diversity and ecosystem functioning: a complex tale of differential effects on decomposition, Oecologia.

## International conference contributions (first author)

**De Meester, N.** & Bonte, D. Dispersal in dwarf spiders: importance of public information and influence on mating and settling. Organisms on the move: On the ecology and evolution of dispersal. 4th meeting of the International Dispersal workgroup, Ghent, 14 -15 September 2009. Poster presentation.

**De Meester, N.,** Derycke, S. & Moens, T. Salinity effects on the competition between cryptic species of the marine nematode *Rhabditis (Pellioditis) marina*, Fourteenth International Meiofauna Conference, Ghent, 11 –16 July 2010. Oral presentation.

**De Meester, N.,** Derycke, S. & Moens, T. Salinity effects on the competition between cryptic species of the marine nematode *Rhabditis (Pellioditis) marina*, Benelux Congress of Zoology, Ghent, 22 - 23 October 2010. Oral presentation.

**De Meester, N.,** Derycke, S. & Moens, T. Same looks, different preferences: salinity effects on the coexistence of cryptic species. Eco-evolutionary dynamics symposium, Leuven, 3 –5 February 2011. Poster presentation.

**De Meester, N.**, Gingold, R., Derycke, S., Rigaux, A. & Moens, T. Functional consequences of nematode cryptic diversity: the case of the *Litoditis marina* species complex. 2nd International Symposium on Nematodes as Environmental Bioindicators. 5 –6 July 2012. Gent. Poster presentation.

**De Meester, N.**, Derycke, S., Monteiro, L.C., Rigaux, A. & Moens, T. Active dispersal to avoid competition? A lab experiment with the *Litoditis marina* (Nematoda) cryptic species complex, Movement and Dispersal Conference, 10 –11 November 2013, Aberdeen. Oral presentation.